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13. ABSTRACT *(Maximum 200*

Overexpression of HER-2/*neu* (a member of the EGF receptor family) and inactivation of estrogen receptor (ER) or the tumor suppressor gene, Rb, are known to be involved in the development of human breast cancer. Expression of HER-2/*neu* can be regulated by ER or Rb. In addition, expression of other EGF receptor family genes (EGF receptor, HER-3 and HER-4) and Heregulin, a ligand for the HER-3 and HER-4 encoded receptor has also been found in some breast cancer cells and may contribute to malignant transformation of breast cancer.The current progress report focuses on the role of the EGF receptor family genes, Heregulin, ER, Rb, and their interrelationship in breast cancer. The technical objectives are: **(1) Systematic studies on the expression of EGF receptor family genes, Heregulin, and ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival; (2) Potential paracrine and autocrine loops for EGF receptor family molecules and Heregulin; (3) Effects of ER on malignant transformation phenotypes breast cancer cells; and (4) Effects of Rb on malignant transformation phenotypes breast cancer cells.** Expression of EGF receptor family genes, Heregulin, and ER in the same breast tumor specimens were examined by immunohistochemical staining, and western blotting. The relationship between expression of these molecules, tumor grades, and patient's survival will be evaluated Using gene transfer technique, Heregulin, ER or Rb gene are being introduced into HER-2/*neu*-expressing breast cancer cells. The effect on their malignant transformations will be examined. *This project may help to develop a more reliable molecular prognostic strategy and to understand how interactions among multiple genetic factors are involved in the development of breast cancer.*

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INTRODUCTION

A. Background

The data from the Surveillance, Epidemiology, and End Results (SEER) Program indicates breast cancer remains a major cause of death in this country (1). It was estimated that approximately 180,000 new cases of breast cancer will be diagnosed in the United States in 1995, and 46,000 women will die from this disease. Many oncogenes and tumor suppressor genes were shown to be involved in the development of breast cancer. The clinical value of each of these genes in prognostic and potential therapeutic applications has been studied to some extent (2). Yet, none of these molecular markers alone was found to be a better prognosis factor than the prognosis factors currently used for breast cancer patients (e.g., number of metastatic lymph nodes). **Identification of a set of related genes that are involved in breast cancer may be critical to develop a better molecular prognostic strategy. Studies on the role of a set of related genes and their interrelationship in breast cancer may also provide a more productive avenue to understand the basic biology of breast cancer cells.** Loss of estrogen receptor (ER) expression or function has been known for a long time to be associated with poor prognosis for breast cancer patients. The *HER-2/neu* oncogene encodes a growth factor receptor-like molecule and overexpression of *HER-2/neu* is also reported to correlate positively with poor survival for breast cancer patients. Some studies, including our own, demonstrate that estrogen-stimulated ER can repress *HER-2/neu* overexpression, suggesting a possible causal relationship between *HER-2/neu* overexpression and non-function of ER in breast cancer cells. The Rb gene is known to be inactivated in approximately 20% of breast tumors. It is not yet clear whether Rb might have prognosis value in breast cancer.

A-1. The *HER-2/neu* Proto-oncogene Encoding an Epidermal Growth Factor (EGF) Receptor-related Protein is a Potent Transforming Oncogene

The rat *neu* oncogene was a transforming gene originally isolated from rat neuroblastoma and later its cellular counterpart, the normal *neu* gene, was also isolated from rat and human libraries (3-6). Detailed structural and functional analysis of the transforming rat *neu* and the normal *neu* genes (*neu* proto-oncogene) indicates that a single point mutation in the transmembrane region is responsible for the conversion of the normal *neu* gene into a transforming *neu* oncogene (7). The human homologue (HER-2 or c-*erb*B-2) of rat *neu* oncogene was isolated based on its homology to chicken v-*erb*B gene (HER-2 represents Human EGF Receptor-2 and c-*erb*B-2 means the second gene homologous to v-*erb*B; EGF receptor gene was the first). It is now known that *neu*, HER-2, and c-*erb*B2 are the same gene. We will use *HER-2/neu* to represent this gene since *HER-2/neu* seems to be the most frequently used in the literature. Structural comparison between the *HER-2/neu*-encoded p185 protein and EGF receptor revealed significant sequence homology and identical gross structural organization including ligand-binding, transmembrane, and tyrosine-kinase domains between these two proteins (5-8). Both EGF receptor and p185 proteins can form either homodimers or heterodimers (9-11). The homodimer is believed to be an active receptor form for ligand binding. Two other EGF receptor-related genes, HER-3 and HER-4, have also recently been cloned. Recent experimental evidence indicates that these four EGF receptor-related proteins are able to form heterodimers among each other, suggesting different combination of heterodimers may interact with distinct ligands and induce signal transduction in specific cell type (12,13). A molecule (named Heregulin or NDF), thought to be a ligand for HER-2/*neu*, recently has been shown to be a ligand for HER-3 and HER-4 (14-19). Heregulin is able to bind HER-3 and HER-4 homodimers but cannot bind to HER-2/*neu* or EGF receptor homodimers. However, Heregulin is able to interact with HER-2/*neu* through heterodimerization of HER-2/*neu* with HER-3 or with HER-4 (12,13). Since Heregulin and the four EGF receptor-related proteins (EGF receptor, HER-2/*neu*, HER-3 and HER-4) can interact with each other, it is likely that they may all be involved in the development of breast cancer.

A-2. Amplification/Overexpression of the Human *HER-2/neu* Gene is Frequently Found in Human Cancers including Breast Cancer.

Unlike the rat *neu* oncogene that is activated by a single point mutation, the human *HER-2*/neu gene is activated by overexpression in human cancers. Amplification/overexpression of the *HER-2/neu* gene was first found in approximately 30% of human breast cancers (20-25) and later in many other human cancers (31-36). In the case of breast, ovarian, lung and gastric cancers, several reports further indicated that *HER-2/neu* overexpression correlates with a poor survival rate (25-31), suggesting that *HER-2/neu*

overexpression may be used as a prognosis factor (25-27, 20, 22). However, some studies disagree that *HER-2/neu* overexpression can be a poor prognostic factor in breast cancer (32-34). Although the discrepancy could be caused by reasons such as patient population and treatment differences, the methods and reagents used for detection of *HER-2/neu* overexpression and the way tumor specimens were collected, other more significant reasons may also contribute to this discrepancy. Considering the fact that the development of breast cancer requires multi-step activations, it is possible that *HER-2/neu* overexpression alone may not be an ideal prognosis factor for breast cancer patients. Combined data from the expression of multiple oncogenes and tumor suppressor genes involved in breast cancer may provide a more accurate prognosis. This point is further addressed in the BODY section of this progress report.

A-3. Non-function of ER May Contribute to *HER-2/neu* Overexpression in Some Breast Cancer

Steroid hormones play an essential part in regulating the growth of both normal and neoplastic breast cells. Specifically, estrogen has a marked effect on the proliferation of breast cells *in vivo* and *in vitro*. Although the mechanisms by which estradiol (E2) induces proliferation in estrogen receptor (ER) positive breast cells are incompletely defined, modulation in expression of certain growth related cellular proto-oncogenes by estradiol stimulated estrogen receptor (E2/ER) has been well-described using cell lines established from human breast tumors. The function of ER in breast cancer is unclear; however, the significant correlation between loss of functional estrogen receptor and poor patient prognosis is very well described (35,36). Evidence that ER may play a role in the regulation of *HER-2/neu* expression comes from several diverse observations. Several studies of human breast tumor tissue specimens have shown an inverse relationship between ER and *HER-2/neu* expression (37-40). Furthermore, during development of rat mammary glands, expression of *HER-2/neu* is inversely related to ER status (41). Those breast cancer cell lines with the highest levels of *HER-2/neu* overexpression are generally ER negative or have very low levels of estrogen receptor. We and others have previously shown that ER through estrogen stimulation can negatively regulate the expression of *HER-2/neu* in ER positive but not ER negative breast cancer cell lines (42-44). We further demonstrated that the ER-mediated *HER-2/neu* repression can occur at the transcriptional level (44). The result suggests that *HER-2/neu* overexpression may be caused by inactivation of ER in some breast tumors. It also raises an interesting possibility that expression of ER in the ER negative and *HER-2/neu*-overexpressing breast cancer cells may suppress malignant transformation induced by *HER-2/neu* overexpression. If this indeed is correct, it may provide an interpretation for a well-known clinical phenomenon, namely, some ER positive breast cancer patients do not respond to hormone treatment such as Tamoxifen (TAM), an estrogen antagonist.

A-4. The Tumor Suppressor Gene, Rb, May Suppress Tumorigenicity of Human Breast Cancer Cells

The retinoblastoma susceptibility gene (Rb) is a well-characterized tumor suppressor gene (45). The existence of this gene was initially predicted based upon genetic predispositions to certain pediatric malignancies (46). Tumor formation or transformation occurs when these genes are inactivated, suggesting that their normal function is to limit cellular proliferation. Inactivation or deletion of Rb has been found in a variety of human cancers including breast cancer (45). Using retroviral-mediated gene transfer, it has been shown that the Rb gene can suppress tumor formation of retinoblastoma, osteosarcoma, and breast carcinoma in which the endogenous Rb gene is inactivated (47-49).

The Rb gene encodes a 105kDa protein (RB) and is known to form a protein complex with adenovirus E1A protein as well as large T (LT) antigen of SV40 virus and E7 protein of papilloma virus (50-52). It is believed that the DNA virus-associated proteins such as E1A, LT and E7 may inactivate the RB function through RB-E1A (or LT, E7) complex. Biochemically, RB can function as a transcriptional factor that can regulate transcription of cellular genes including c-*myc*, TGF-b, c-*fos* and *HER-2/neu* (53-56). More recently, RB has been shown to form a protein complex with a DNA-binding protein E2F and may, therefore, act as a transcriptional factor by complexing with other factors (57,58).

B. THE PURPOSES

The major purposes are:

B-1. Systematic studies on the expression of EGF receptor family, Heregulin, ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival.

Our hypothesis is that the combination of EGF receptor family, Heregulin, ER may be a better prognosis factor than each of these molecules individually. Therefore, expression of EGF receptor family, Heregulin, ER, in the same breast tumor specimens will be examined by immuno-histochemical staining. The relationship between expression of these molecules, tumor grades and patients' survival will be evaluated.

B-2. Potential paracrine and autocrine interactions between EGF receptor family and Heregulin in breast cancer cells.
Potential paracrine and autocrine loops between EGF receptor family and Heregulin ligand will be tested by using expression vectors and model cell lines. Effects of Heregulin on transformation phenotypes of breast cancer cells will be examined by growth properties, soft agar colonization assay, subcutaneous (s.c.) tumorigenicity and intraperitoneal (i.p.) survival assays. The Heregulin-mediated signal pathway will be studied, especially emphasize on involvement of NF-kB.

B-3. Effects of ER on malignant transformation phenotypes of *HER-2/neu*-overexpressing breast cancer cells.
Since we have found that estrogen-stimulated ER can repress *HER-2/neu* gene expression, ER expression vectors will be used to modulate *HER-2/neu* expression in *HER-2/neu* overexpressing breast cancer cells. The effect of ER on transformation phenotypes of *HER-2/neu* overexpressing breast cancer cells has been examined. The effect of receptor tyrosine kinase on ER will also be examined.

B-4. Effects of Rb on malignant transformation phenotypes of breast cancer cells.
The effects of Rb on *HER-2/neu* expression and transformation phenotypes have been analyzed. The effect of RB-associated E2F-1 on breast cancer cells will be explored.

BODY

1. Systematic studies on the expression of EGF receptor family, Heregulin, ER and Rb.

As mentioned in the progress reports of 1996 and 1997, Heregulin is not a ligand for HER-2/*neu* (12-14). Instead, Heregulin is a ligand for HER-3 and HER-4 (also known as c-*erb*B3 and c-*erb*B4) that are two recently identified EGF receptor family genes. Since HER-3 and HER-4 can form heterodimers with HER-2/*neu* and interactions between Heregulin and HER-2/*neu* is most likely through HER-3:HER-2/*neu* dimer or HER-4:HER-2/*neu* dimer (12-13), it is possible that HER-3 and HER-4 may also contribute to breast cancer. In addition, overexpression of EGF receptor is known to be involved in human breast cancer. In the 1997 Progress Report, we have examined the expression of these genes in a number of breast tumor tissues for all 4 EGF receptor family (EGF receptor, HER-2/neu, HER-3 and HER-4).

In the current report, We have used specific antibodies for EGF receptor, HER-2/*neu*, HER-3, HER-4, and Heregulin to stain the archival paraffin-embedded sections. Up to now, the examination has been expeanded to 300 tumor sections, as we proposed in last year, for all EGF receptor family members including the EGF receptor, HER-2/*neu* , HER-3 and HER-4. One set of representative data was shown in Fig. 1. In these cases, we can identify tumors with high, intermediate, low and negative expression. Quantitation of antibody staining is justified by relative intensity of positive staining by professional pathologist. It is obvious that differential expression of all four EGF receptor family can be found in the tumor tissues, suggesting their possible role in the development of breast tumors. We have collected the information for the clinical status including tumor grades, patient survival, lymph node metastasis, and ER status from the archival medical record. Currently, we are analyzing the relationship between clinical status and expression of each individual gene and a set of genes. Multiple variant analysis is being conducted. Recently we have extended the study to a small set of oral cancer patients. An example analysis derived from 47 cancer patients is shown in Table 1, in which we show that the combination of expression levels of the EGFR family members can result in higher predicting power of the outcome of the cancer patients in terms of tumor histological grades, and node and distant metastasis. Currently we are using a similar approach to analyze the data derived from breast cancer patients.

As mentioned in previous progress reports, immunohistochemical staining for Rb protein will not be a very informative strategy to detect inactivation of Rb in tumor tissues (This was also discussed in the original grant proposal.). We therefore focus on EGF receptor family and Heregulin in the expression studies.

2. Potential Paracrine and Autocrine Interactions between HER-2/*neu* and Heregulin in Breast Cancer Cells.

In addition to our original approaches to establish stable transfectants of Heregulin in HER-2/*neu* and HER-3 positive breast cancer cell lines and anti-sense Heregulin in Heregulin-expressing cells (such as MDA-MB-231), we have also used another approach by adding Heregulin to the HER-2 and HER-3 positive cells. We used breast cancer cells, MCF7 (HER-3 positive) and its HER-2/*neu* transfectants, MCF-7/HER-2-11 and MCF-7/HER2-18 (HER-3 positive and HER-2/neu overexpression) and found that upon Heregulin stimulation, NF-kB is activated in the transfectants (HER-3, HER-2 positive), but not in the parental MCF7 cells (HER-3 positive and HER-2/*neu* basel level) (Fig. 2), and this observation was correlated with increased cell survival (Fig. 3). Activation of NF-kB has been shown to have anti-apoptic effect. The results shown in Figs 2 and 3 suggest that Heregulin may stimulate NF-kB and this activation requires HER-2/*neu* overexpression (mostly through HER-3/HER-2 heterdimer). The Heregulin-mediated NF-kB activation may produce anti-apoptotic activity and contribute to Heregulin-stimulated cell growth. This may provide a molecular mechanism for paracrine and/or autocrine interactions between HER-2/*neu* and Heregulin in breast cancer cells. A systematic study on this issue is currently undergoing using MTT assay, thymidine incorporation assay and apoptotic assays including DNA ladders, TUNEL assay etc in the MCF7 and its HER-2/*neu* transfectants.

3. Effects of ER on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells.

In the last report, we have shown that our hyposthesis is correct that estrogen suppresses transformation phenotype of ER^+, HER-2/*neu* -overexpressing breast cancer cells. A reprint of the published paper (Oncology Report) was attached in the previous report. It is known that HER-2/*neu* and its family receptors are tyrosine kinase receptors. In this report, we have extended our study on the involvement of tyrosine kinase pathway and proteosome-mediated pathway in the regulation of ER signaling. Part of this work has been described at the 1997 AACR annual meeting. A manuscript is under preparation and will be submitted to the Journal of Biological Chemistry (a draft is attached). The key data of the manuscript are described as following and shown as Fig 4-9. These results have provided a linkage between ER and tyrosine kinase signal pathways.

In an attempt to study the involvement of tyrosine kinase pathway in estrogen receptor signaling pathway, we used emodin, a tyrosine kinase inhibitor, to treat MCF-7 breast cancer cell line which express high level of estrogen receptor protein. Here we reported that emodin inhibited estrogen-induced expression of bcl-2 protein in MCF-7 breast cancer cells. Unexpectedly, treatment of the cells with emodin rapidly depletes cellular levels of estrogen receptor protein in a dose- and time-dependent manner. The pulse chase experiment showed that the decrease was resulted from enhanced degradation of estrogen receptor protein, not the rate of synthesis. To examine the mechanism involved in the emodin-enhanced degradation of estrogen receptor, inhibitors of the lysosomal (chloroquine), proteasome (PSI and MG115), and calpains pathways were used. We found that only PSI and MG115, which specifically inhibit the chymotrypsin-like activity of proteasome, blocked emodin induced depletion of estrogen receptor protein levels. The results suggest that the proteasome proteolytic pathway may be involved in the emodin-induced decreases in estrogen receptor protein levels. We then examined the effect of emodin on the hsp90-estrogen receptor heteromeric complex formation. We found that there was a marked increase in the complex formation. The data demonstrate that emodin may inhibit the dissociation of hsp90 from estrogen receptor, resulting in the degradation of estrogen receptor. We have tested other tyrosine kinase inhibitors (RG13022 and genistein) and similar results were observed (data not shown). All together these findings indicate that proteasome-mediated protein degradation can modulate estrogen receptor protein level, hsp90 can mediate the degradation of the estrogen recetor, and the possible use of emodin and other tyrosine kinase inhibitors in the therapeutic manipulation of this process.

4. Effects of Rb on Malignant Transformation Phenotypes of Breast Cancer Cells

Using adenoviral vector expressing Rb, we have shown in the last report that Wt Rb can suppress transformation phenotype of Rb-defective breast cancer cells such as MDA-MB-468 and BT-549. Since RB is known to bind to E2F-1, we extend our study to examine effect of E2F-1 overexpression on breast cancer cells. We have made an adenoviral vector that express E2F-1 and found that overexpressing of E2F-1 induces apoptosis in human breast and ovarian cancer cells. A manuscript describing these works have been published in Cancer Research (Hunt et al., Cancer Res. 57:4722-4726, 1997).

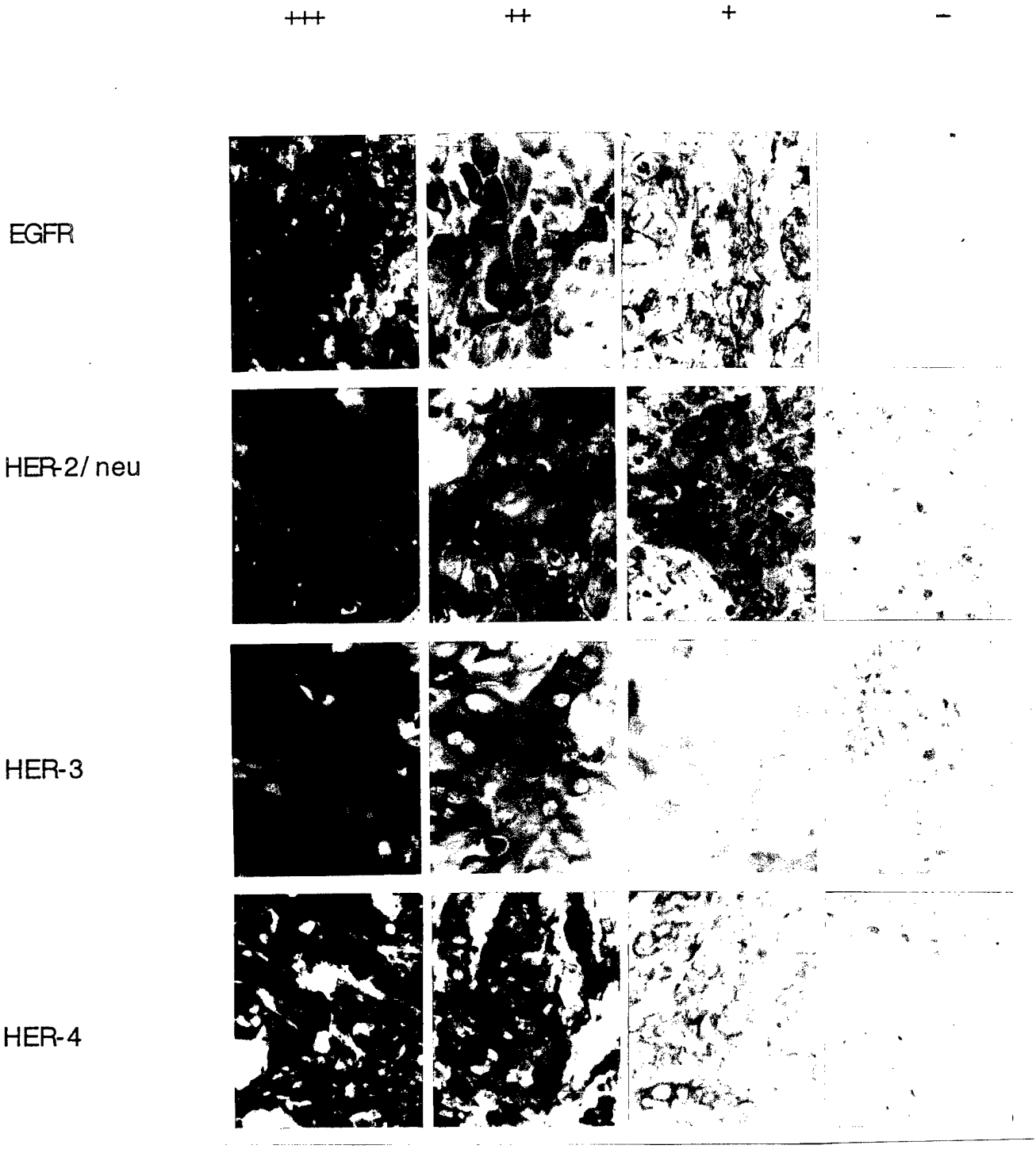

Fig.1. Immunohistostaining of tumor sections for EGF recepter family. The relative intensities were marked by +++, ++, +, and - .

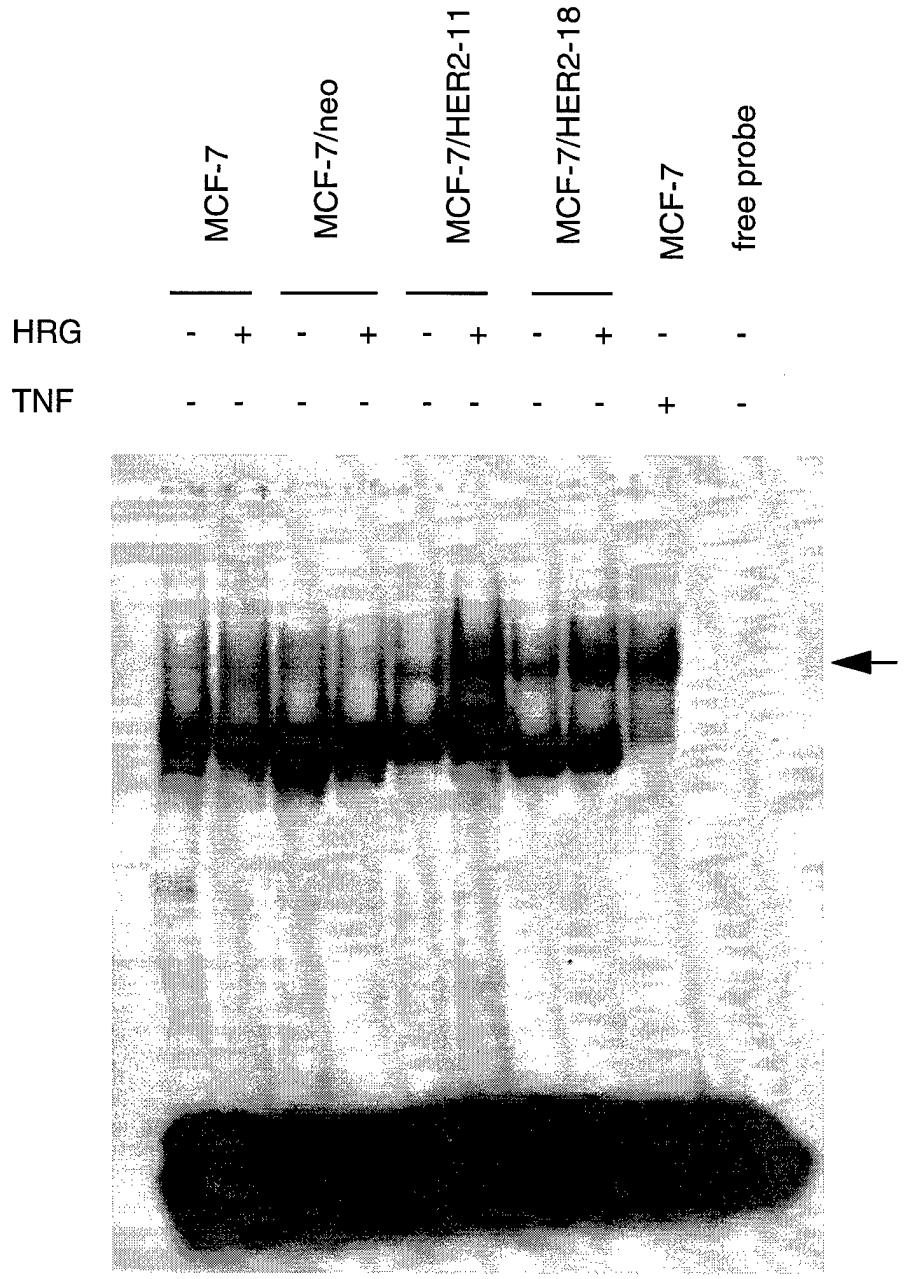

Fig. 2 Heregulin activates NF-κB in MCF-7 cells overexpressing HER-2/neu. Cells were treated in presence or absence of heregulin (5ng/ml) for 1 hr in PBS/ 0.1% BSA. Equal amounts of nuclear extracts were subjected to electrophoretic mobility shift analysis to detect NF-κB DNA binding activity. MCF-7/neo, MCF-7 transfected with vector; MCF-7/HER2-11 and MCF-7/HER2-18, two transfectant cell lines overexpressing HER-2/neu. Arrow indicates p50/RelA complex bound to oligonucleotides containing κB site.

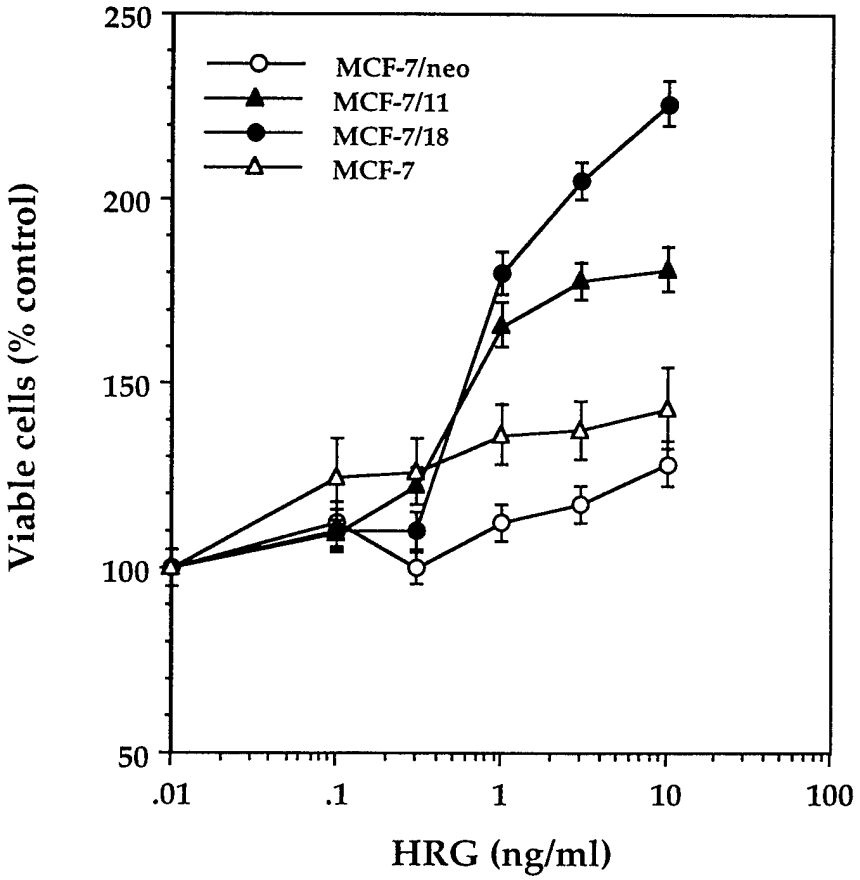

Fig. 3 Heregulin stimulates cell growth/survival in MCF-7 transfectants overexpressing HER-2/neu. Cells were seeded at 2 x 10^4 cells/ml in a 96-well microtiter plate overnight in 10% serum medium. Subsequently cells were incubated for additional 72 hrs in a medium containing 0.1% serum and varying amounts of heregulin. Cell viability was measured by MTT assay.

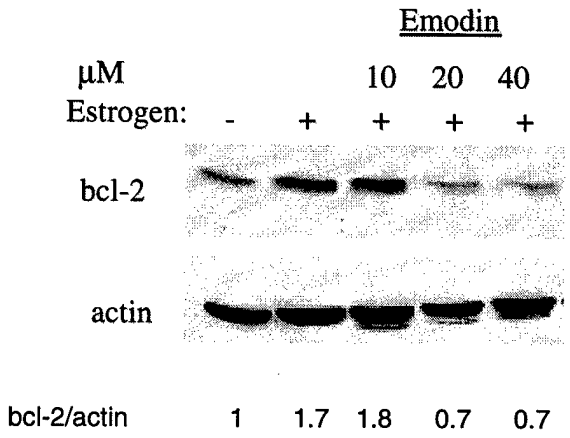

Fig. 4. **Emodin inhibits estrogen-induced bcl-2 protein expression in MCF-7 cells.** MCF-7 cells were grown in the estrogen depleted medium for one day before the initiation of the experiment. The cells were then stimulated with estrogen (10 nM). Different concentrations of emodin were added simultaneously. After 24 hours, total cell lysates were prepared and immunoblotting analyses for bcl-2 protein were determined. The membrane was stripped and reprobed with anti-actin antibody to show the protein loading.

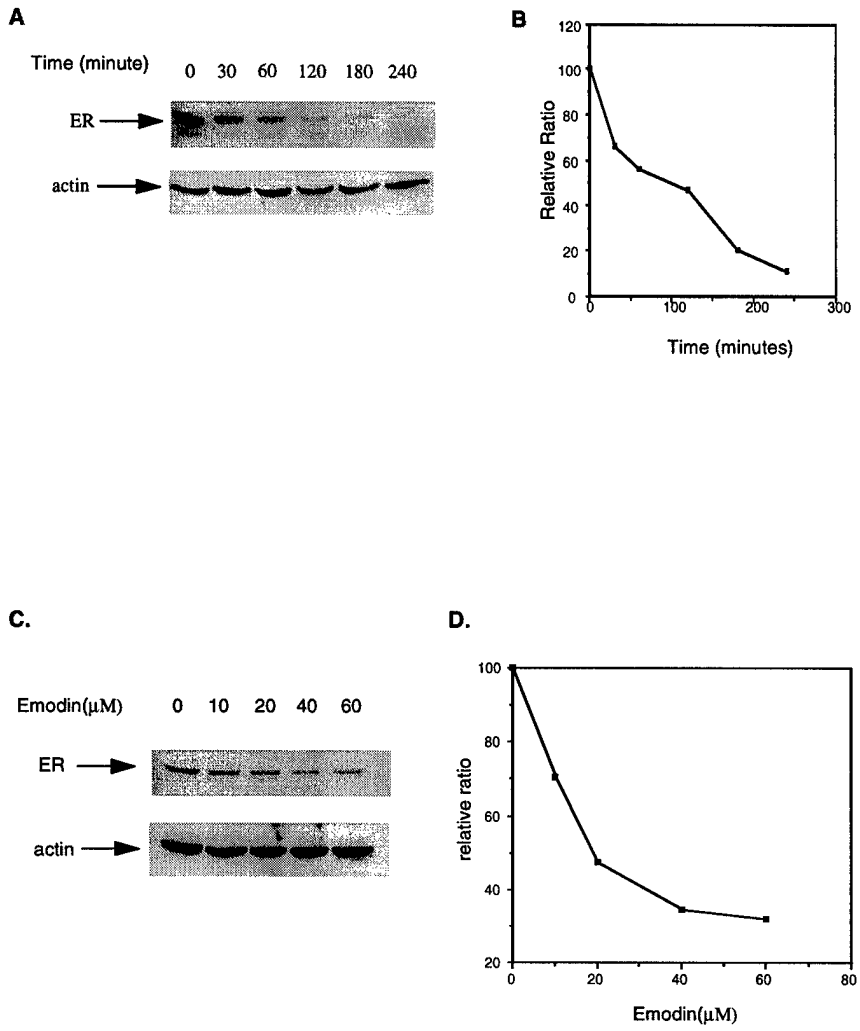

Fig. 5. **Depletion of estrogen receptor protein in estrogen receptor positive cells by emodin.** *A*, MCF-7 cells were exposed to 40 μM emodin for various time intervals and extracted as described in "Experimental Procedures". Estrogen receptor protein levels in MCF-7 cells were measured by immunoblotting with monoclonal antibody D75. The same membrane was stripped and reprobed with anti-β-actin antibody to show the protein loading. *B*, the proteins were quantitated by NIH Image software and plotted as the percentage control (without emodin) and normalized with actin. C, MCF-7 cells were incubated with different concentrations of emodin for 4 hours. The protein levels were then examined by immunoblotting as described above. The protein levels were quantitated as described above and plotted as showed in panel D.

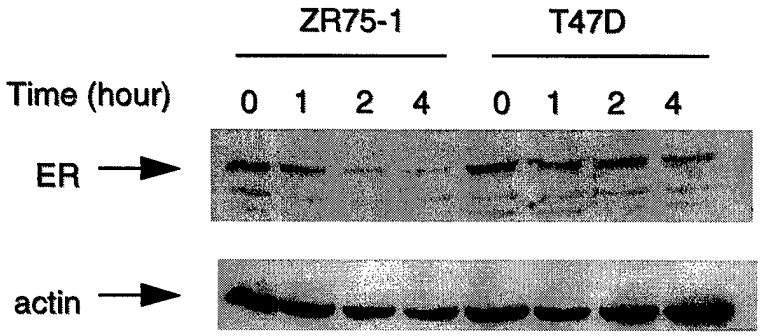

Fig. 6. Effect of emodin on estrogen receptor protein levels was examined in two other estrogen receptor positive cell lines, ZR75-1 and T47D. The cells were treated with 40 μM emodin for different time intervals. Immunoblotting analysis for estrogen receptor protein was performed. The membrane was stripped and reprobed with anti-actin antibody to show the protein loading.

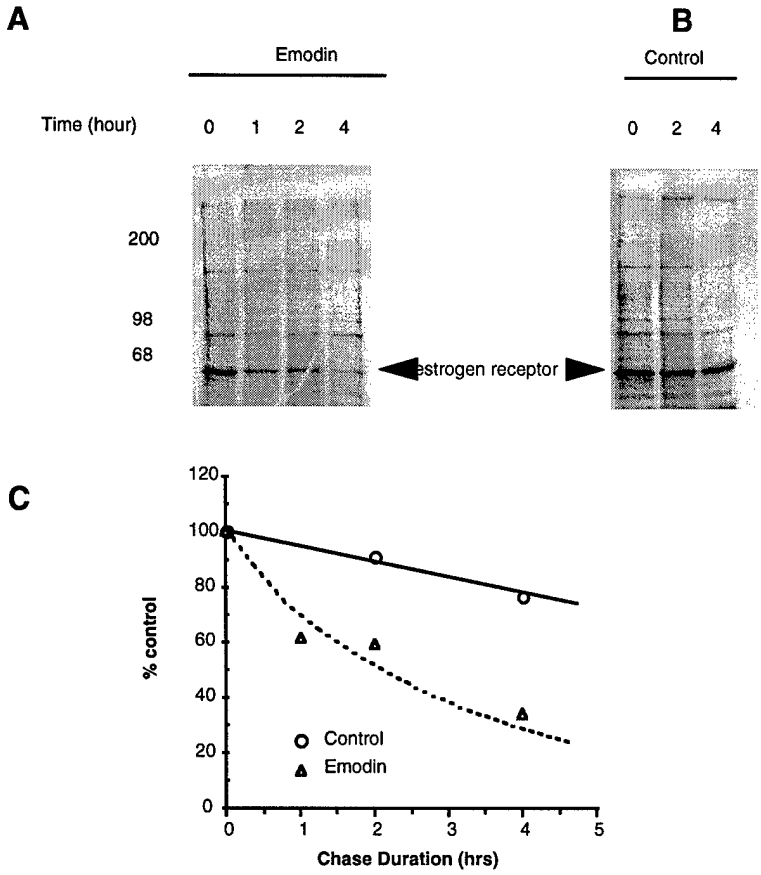

Fig. 7. **Emodin-enhanced estrogen receptor protein degradation.** Pulse chase experiment was performed to determine the stability of the estrogen receptor proteins after treatment with emodin (see "Experimental Procedures" for details). *A*, MCF-7 cells were treated with emodin at different time intervals. *B*, MCF-7 cells were treated with DMSO at various time intervals. *C*, proteins were quantitated by NIH Image software and plotted as the percentage of the value at the beginning of the chase.

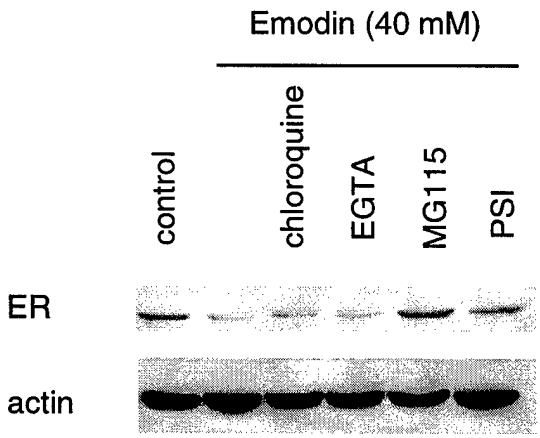

Fig. 8. **Effect of different protease inhibitors on emodin-induced estrogen receptor protein degradation.** Chloroquine (100 μM), EGTA (5 mM), MG115 (25 μM), and PSI (25 mM)were added to MCF-7 cells simultaneously with 40 mM emodin at several time intervals. PBS and DMSO were added to the control. The cells were then harvested and the expression levels of estrogen receptor protein were measured by western blotting analysis.

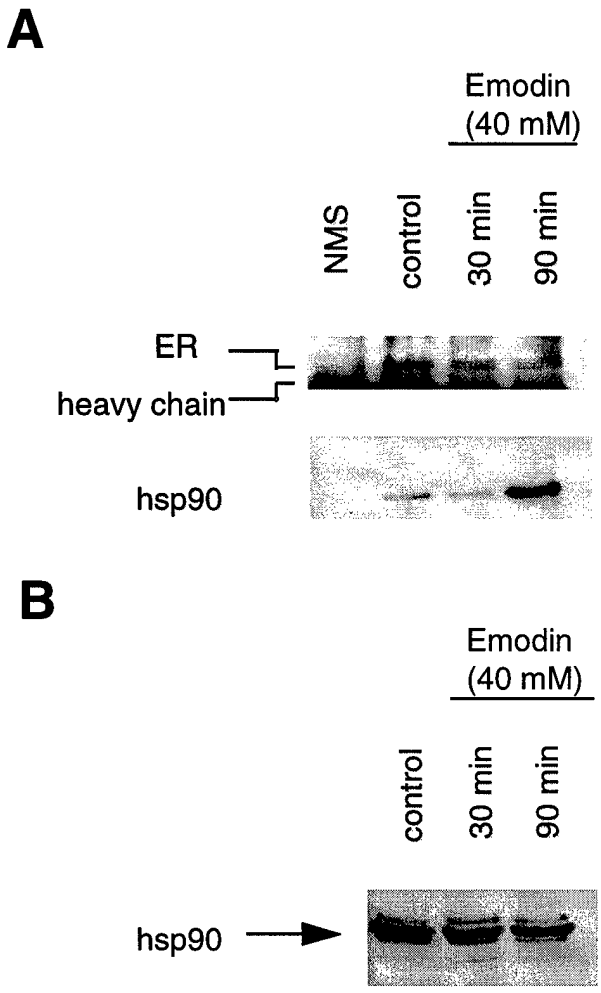

Fig. 9. **Enhanced association of hsp90 and estrogen receptor protein in MCF-7 cells after incubation with emodin.** Estrogen receptor immunoprecipitates (by anti-estrogen receptor antibody, SRA1010) from MCF-7 cells were analyzed by SDS/PAGE and immunobloting with anti-hsp90 (AC88). Normal mouse serum (NMS) was used instead of anti-estrogen receptor antibody as a control. The membrane was then stripped and reprobed with anti-estrogen receptor (D75) antibodies (*A*). The same protein lysates were used to examine the hsp90 protein level by immunoblotting (*B*).

Table 1. *Results of univariate and multivariate analyses using the Cox regression analysis model*

Univariate[a]			Multivariate[b]								
	log likelihood ratio	partial[c] *p* value	Combination of two factors	log likelihood ratio	partial *p* value	Combination of three factors	log likelihood ratio	partial *p* value	Combination of all four factors	log likelihood ratio	partial *p* value
HER-2/neu	-19.20	< 0.001	HER-2/neu +HER-3	-13.94	0.002	HER-2/neu +HER-3 +EGFR	-11.99	< 0.05	HER-2/neu +HER-3 +EGFR +HER-4	-11.66	> 0.25
HER-4	-19.54	< 0.001	HER-2/neu +HER-4	-16.84	< 0.05	HER-2/neu +HER-3 +HER-4	-13.82	> 0.5			
HER-3	-20.72	< 0.001	HER-2/neu +EGFR	-18.35	> 0.25						
EGFR	-25.75	< 0.001									

[a] Addition of single oncoprotein to Cox regression analyses which includes histological grade, nodes, and distant metastasis.
[b] Multivariate addition of oncoproteins to Cox regression analysis which includes histological grades, nodes, and distant metastasis.
[c] Log likelihood relative to log likelihood of -36.0 for grade, nodal stage, and distant metastasis

CONCLUSION:

Task 1: Screening of 300 tumor sections for EGF receptor, HER-2/*neu* , HER-3, and HER-4 has been completed. Due to the complexity caused by the newly identified HER-3 and HER-4 (12, 13), we have focused more on the staining of EGF receptor, HER-2/*neu*, HER-3, HER-4 and Heregulin. We have collected patient information and is currently analyzing the relationship between expression of the HER genes and patient clinical status from medical record.

Task 2: Heregulin was found to activate NF-kB activity in the HER-3 positive and HER-3/*neu*-overexpressing cells but not in the HER-3 positive and HER-2/*neu* low-expressing cells.

Task 3: Characterization of E2 on ER$^+$, HER-2/*neu*-overexpressing breast cancer cells was completed. A manuscript describing this part of work was published and reported previously. A group of tyrosine kinase inhibitors were found to inactivate ER through depletion of ER via proteosome proteolytic pathway. A manuscript describing the regulation of ER by the tyrosine kinase inhibitor-sensitive pathway will be submitted soon. The manuscript also describes the proteosome-involved mechanism in ER downregulation by tyrosine kinase inhibitors.

Task 4: Expression of Rb is able to suppress transformation phenotype of Rb-defective breast cancer cells. The Rb-associated E2F-1 protein was found to induce apoptosis in breast cancer cells through a p53-independent pathway.

In addition to the those described in the BODY Section, several studies relating to the *HER-2/neu* oncogene in cancer have been completed. The funding support from the current project has been appropriately acknowledged in the resulting publications (reprints or manuscript are attached). These include:

1. Lisha Zhang, Yiu-Keung Lau, Larry Xi, Ruey-Long Hong, Darrick SHL Kim, Chieh-Fu Chen, Garbriel N Hortobagyi, Ching-jer Chang, and Mien-Chie Hung. Tyrosine kinase inhibitors, emodin and its derivative repress *HER-2/neu*-induced cellular transformation and metastasis-associated properties. *Oncogene* 16, 2855-2863, 1998.

2. Nobutaka Kiyokawa, Devarajan Karunagaran, Eun Kyung Lee, Youming Xie, Deun-Hwa Yan, and Mien-Chie Hung. Involvement of cdc2-mediated phosphorylation in the cell cycle-dependent regulation of p185^{neu}. *Oncogene* 15, 2633-2641, 1997.

3. Yiu-Keung Lau and Mien-Chie Hung. Degradation of estrogen receptor by emodin, a tyrosine kinase inhibitor, through proteasome pathway. To be submitted, 1998.

Those resulted publications reported in the 1997 Progress Report are listed below (reprints are not attached as they have been reported before):

1. Kelly K. Hunt, Jiong Deng, Ta-Jen Liu, Marcia Wilson-Heiner, Stephen G. Swisher, Gary Clayman, and Mien-Chie Hung. Adenovirus-Mediated Overexpression of the Transcription Factor E2F-1 Induces Apoptosis in Human Breast and Ovarian Carcinoma Cell Lines and Does Not Require p53[1]. *Cancer Research* 57:4722-4726, 1997.

2. Naoto T. Ueno, Dihua Yu, and Mien-Chie Hung. Chemosensitization of HER-2/neu-overexpressing human breast cancer cells to paclitaxel (Taxol) by adenovirus type 5 E1A. *Oncogene* 15:953-960, 1997.

3. Nobutaka Kiyokawa, Eun Kyung Lee, Devarajan Karunagaran, Shiaw-Yih Lin, and Mien-Chie Hung. Mitosis-specific Negative Regulation of Epidermal Growth Factor Receptor, Triggered by a Decrease in Ligand Binding and Dimerization, Can Be Overcome by Overexpression of Receptor. *J. Biol. Chem.* 272(30):18656-18665, 1997.

4. Joe Yujiao Chang, Weiya Xia, Ruping Shao, Frank Sorgi, Gabriel N. Hortobagyi, Leaf Huang, and Mien-Chie Hung. The tumor suppression activity of E1A in HER-2/neu-overexpressing breast cancer. *Oncogene* 14:561-568, 1997.

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6. Youming Xie and Mien-Chie Hung. $p66^{Shc}$ Isoform Down-Regulated and Not Required for HER-2/*neu* Signaling Pathway in Human Breast Cancer Cell Lines with HER-2/*neu* Overexpression. *Biochem. Biophys. Res. Comm.*221:140-145, 1996.

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NOTE: Please find also attached the abstract from the 1997 Era of Hope Meeting sponsored by The Department of Defense Breast Cancer Research Program.

THE TUMOR SUPPRESSION ACTIVITY AND CHEMOSENSITIZATION OF E1A IN HER-2/NEU-OVEREXPRESSING BREAST CANCER

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Amplification or overexpression of *HER-2/neu* has been demonstrated in human breast cancer, and correlated with chemoresistance and shorter survival. In previous work, we showed that the adenovirus type 5 early region 1A (E1A) gene product can repress *HER-2/neu* overexpression by repressing *HER-2/neu* promoter activity, and suppress the tumorigenic potential of *HER-2/neu*-overexpressing ovarian cancer cells. To examine E1A tumor suppressor function in breast cancer, we transduced E1A *in vitro* by adenovirus into both *HER-2/neu*-overexpressing and low expressing human breast cancer cell lines. In *HER-2/neu*-overexpressing cells, E1A greatly inhibited tumor cell growth *in vitro*. However, in low *HER-2/neu* expressing cancer cell lines, E1A had no significant effect on cell growth in culture medium. To test the therapeutic efficacy of E1A, we used both adenovirus-mediated and cationic liposome-mediated E1A gene delivery systems in an orthotopic breast cancer animal model. An advanced breast cancer model was established by inoculation of *HER-2/neu*-overexpressing human breast cancer cells into mammary fat

Keywords: *HER-2/neu*, E1A, Gene Therapy, Chemosensitization, Breast Cancer

This research was supported by Army Breast Cancer Grant 17-94-J4315

pad and treated by local injections of either replication-deficient adenovirus expressing E1A, Ad.E1A(+) or a liposome-E1A DNA complex. As controls, mice bearing tumors were also treated with Ad.E1A(-), in which E1A is deleted, a liposome-E1A frame-shift mutant DNA complex, or just PBS. In mice bearing a *HER-2/neu*-overexpressing breast cancer cell line, E1A delivered either by adenovirus or liposome significantly inhibited tumor growth and prolonged mouse survival compared with the controls (Fig. 1). 60-80% of E1A-treated mice lived longer than 2 years versus only 0-20% of control mice ($P<0.05$). Immunohistochemical analysis showed that *HER-2/neu* p185 protein expression was suppressed. Since *HER-2/neu* overexpression is known to induce chemoresistance, we also examine whether E1A can sensitize *HER-2/neu*-overexpressing human breast cancer cells to paclitaxel through E1A-mediated *HER-2/neu* repression. An adenoviral vector was used to transfer the E1A gene into two human breast cancer cell lines that overexpress HER-2/*neu*. Cell proliferation assays showed a synergistic growth inhibition effect of E1A and paclitaxel that was confirmed by soft agar colony-formation assay (Fig. 2). Breast cancer cell lines that express low levels of *HER-2/neu* showed no synergistic growth inhibition effect when treated on the same protocols. Taken together, our results indicated that both adenovirus and cationic liposome delivery systems were effective in transfering E1A gene for tumor suppression in a *HER-2 neu*-overexpressing breast cancer model. E1A gene can sensitize paclitaxel-resistant *HER-2/neu*-overexpressing breast cancer cells to the drug by repressing *HER-2 neu* expression.

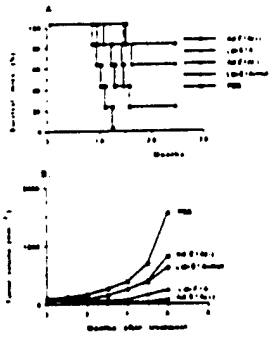

Fig. 1. A. Survival in mice bearing *HER-2/neu*-overexpressing breast cancer. *HER-2/neu*-overexpressing MDA-MB-361 cells (5×10^6) were injected into mammary fat pad in female nu/nu mice (n=10). 2 months later, when the palpable solid tumors were detected, the mice were given a local injection of either 0.2 ml of viral solution ($5X10^8$ PFU/ml) or liposome-DNA complexes once a day for 3 consecutive days, then once a week for six months. The tumor volume was monitored monthly for 6 months. Then, after the treatment was stopped for two months, five mice bearing MDA-MB-361 were sacrificed to detect metastasis, others were observed for survival for up to 2 years. B. Tumor growth in mice bearing *HER-2/neu*-overexpressing breast cancer (n=10).

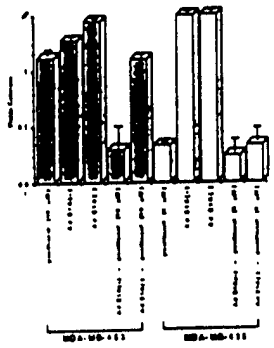

Fig. 2. Effect of E1A plus paclitaxel treatment on soft agar colony formation by *HER-2/neu*-overexpressing breast cancer cell line MDA-MB-453 and low *HER-2/neu*-expressing cell line MDA-MB-431. Bar, SD. The percentage of colonies was calculated by defining the number of colonies formed by cells not treated with paclitaxel and E1A as 100%. Treatment with Ad.E1A(+) (V/T 200:1) and paclitaxel (10 μM) had a synergistic inhibitory, anchorage-independent effect on colony formation by MDA-MB-453 cells. Treatment with Ad.E1A(+) (V/T 200:1) plus paclitaxel (1 μM) had no synergistic effect on MDA-MB-435 colonies. The paclitaxel concentration of 1 μM was chosen because of the MDA-MB-435 line is more sensitive to paclitaxel.

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Tyrosine kinase inhibitors, emodin and its derivative repress *HER-2/neu*-induced cellular transformation and metastasis-associated properties

Lisha Zhang[1], Yiu-Keung Lau[1], Larry Xi[1], Ruey-Long Hong[1,2], Darrick SHL Kim[3], Chieh-Fu Chen[4], Gabriel N Hortobagyi[5], Ching-jer Chang[3] and Mien-Chie Hung[1]

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We have previously shown that emodin suppresses tyrosine kinase activity of *HER-2/neu*-encoded p185^{neu} receptor tyrosine kinase. In this study, we examine the relationship between the chemical structure and the activity of emodin and nine derivatives, and identified that one methyl, one hydroxy, and one carbonyl functional groups are critical for the biological activities of emodin. We also found that one of the derivatives 10-(4-acetamidobenzylidene)-9-anthrone (DK-V-47) is more effective than emodin in repressing the tyrosine phosphorylation of p185^{neu} and in inhibiting the proliferation and transformation of *HER-2/neu*-overexpressing human breast cancer cells. Using mutation-activated *HER-2/neu* transformed 3T3 cells, we also investigated whether emodin and DK-V-47 can inhibit malignant transformation induced solely by the *HER-2/neu* oncogene. We found that DK-V-47 is more potent than emodin in suppressing transformation phenotypes of activated *HER-2/neu* transformed 3T3 cells including anchorage-dependent and -independent growth, metastasis-associated properties. These results clearly indicate that the inhibition of p185^{neu} tyrosine kinase by both emodin and DK-V-47 is capable of suppressing the *HER-2/neu* associated transformed phenotypes including the ability to induce metastatic potential. Our results also support the chemotherapeutic implications of the use of either emodin or DK-V-47 to target *HER-2/neu*-overexpressing cancer cells.

Keywords: emodin; anthraquinone; *HER-2/neu*; tyrosine kinase; experimental metastasis

Introduction

The *HER-2/neu* gene (also known as c-*erbB-2*) encodes a 185^{neu} transmembrane tyrosine kinase (p185^{neu}) homologous to epidermal growth factor receptor (EGFR) (Hung *et al.*, 1986; Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Semba *et al.*, 1985; Yamamoto *et al.*, 1986). Amplification and/or overexpression of *HER-2/neu* has been detected frequently in many human cancers (Slamon *et al.*, 1987, 1989; Zhang *et al.*, 1989; Schneider *et al.*, 1989; Weiner *et al.*, 1990; Yokota *et al.*, 1988; Zhau *et al.*, 1990; Hou *et al.*, 1992; Myers *et al.*, 1994). Its enhanced expression is correlated with chemotherapeutic drug resistance in some experimental systems (Yu *et al.*, 1996; Tsai *et al.*, 1993, 1995), number of lymph node metastases in node positive breast cancer patients (Slamon *et al.*, 1987; Lacroix *et al.*, 1989; Tauchi *et al.*, 1989) and shortened survival in breast, ovarian, lung, oral and gastric cancer patients (Sadasivan *et al.*, 1993; Veltri *et al.*, 1994; McCann *et al.*, 1991; Vijver *et al.*, 1987; Gusterson *et al.*, 1992; Toikkanen *et al.*, 1992; Berchuck *et al.*, 1990; Xia *et al.*, 1997; Yoshida *et al.*, 1989).

Cellular and animal experiments have shown that an increase in *HER-2/neu* expression enhances malignant phenotypes of cancer cells, including metastatic potential (Yu and Hung, 1991; Yu and Hung, 1991; Yu *et al.*, 1994; Yusa *et al.*, 1990). The association of *HER-2/neu* overexpression in cancer cells with chemoresistance and metastasis provides a plausible interpretation for the poor clinical outcome of patients with *HER-2/neu*-overexpressing cancers; it suggests that enhanced tyrosine kinase activity of *HER-2/neu* plays a critical role in the initiation, progression, and outcome of human tumors.

Emodin (3 methyl-1,6,8 trihydroxyanthraquinone) is an inhibitor of protein tyrosine kinase (Jayasuriya *et al.*, 1992) which was isolated from *Polygonum Cuspidatum*. Emodin was recently shown to suppress *HER-2/neu* tyrosine kinase activity in *HER-2/neu*-overexpressing human breast and lung cancer cells (Zhang *et al.*, 1995; Zhang and Hung, 1996). It has also been shown to sensitize *HER-2/neu*-overexpressing lung cancer cells to chemotherapeutic drugs (Zhang and Hung, 1996).

In an attempt to search for compounds more effective than emodin to inhibit *HER-2/neu* tyrosine kinase activity and cell transformation, we examined the relationship between the chemical structure and activity of the derivatives of emodin on tyrosine phosphorylation of *HER-2/neu*. We found that one of nine of the derivatives, 10-(4-acetamidobenzylidene)-9-anthrone (DK-V-47) is more effective than emodin in repressing tyrosine phosphorylation of p185^{neu}, in inhibiting growth, and in suppressing the transformation phenotypes of these cancer cells. Because it is not clear whether emodin and DK-V-47 might also exhibit other biological activity which could also contribute to the suppression of the malignance of human cancer

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cells, it is critical to determine whether the two compounds can inhibit malignant transformation induced solely by the *HER-2/neu* oncogene. To address this issue, we included *HER-2/neu* transformed 3T3 cells in our study.

Results

Effect of emodin and its derivatives on HER-2/neu-*overexpressing human breast cancer MDA-MB 453 cells*

To determine the relationship between chemical structures of emodin and its derivatives and their inhibitory activities on the tyrosine phosphorylation of *HER-2/neu* and on the proliferation of *HER-2/neu*-overexpressing breast cancer cells, we synthesized nine derivatives as shown in Figure 1. We separated the derivatives of emodin into four groups, according to substitute at the different structural position of emodin. We treated human breast cancer MDA-MB 453 cells that overexpress $p185^{neu}$ with varying concentrations of ten compounds at 37°C for 24 h in the absence of serum. We then analysed the ten compounds to determine the protein level of $p185^{neu}$ and its tyrosine phosphorylation, we used immunoblotting with anti-phosphotyrosine antibody for the detection of phosphotyrosine (anti-PY) and anti-$p185^{neu}$ antibody for the detection of $p185^{neu}$. As summarized in the Table 1, of the ten compounds tested the carbon 10 (C_{10}) group substitute DK-V-47 was revealed to be the most effective in suppressing the tyrosine phosphorylation of $p185^{neu}$; the parent compound emodin is slightly less effective. To achieve a 50% inhibitory activity of tyrosine phosphorylation, 21 μM of emodin and 17 μM of DK-V-47 are required. Emodin and its nine derivatives under the same condition did not affect the protein levels of $p185^{neu}$ (data not shown).

We also investigated the effects of emodin and its derivatives on the proliferation of MDA-MB 453 cells. Cells were treated with different concentrations of the compounds at 37°C for 72 h and then measured by the MTT assay. As also shown in Table 1, DK-V-47 is the most effective compound inhibiting cell growth; the IC_{50} of emodin is fivefold higher than that of DK-V-47.

The findings from four groups also indicate that the methyl group at C_3, the hydroxy group at C_6 and the carbonyl group at C_{10} position are critical for maintaining the activity of emodin required to suppress the tyrosine phosphorylation of *HER-2/neu* and to block the growth of *HER-2/neu*-overexpressing breast cancer cells. However, when the ketone at C_{10} is replaced by the *p*-acetamidobenzylidene group, the compound DK-V-47 demonstrates more potent activity than does emodin. The CH_3CO group in the backbone of DK-V-47 appear to be critical for the biological activity; replacement of this group by H almost completely abolishes the biological activities.

We have previously shown that, among the different biological assays, suppression by emodin is much more profound in the soft agarose colonization assay; this assay which is used to measure the ability of cells to grow in an anchorage-independent environment, represents transformation status of cells *in vitro*. As shown in Figure 2, when DK-V-47 was examined for its effect on breast cancer cells in a soft agarose

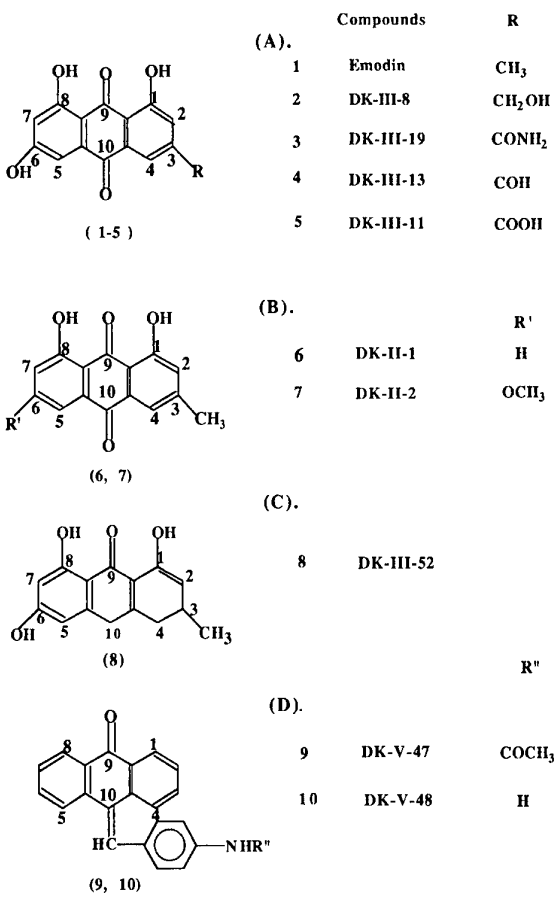

Figure 1 The chemical structures of emodin and its derivatives

Table 1 Relationship between chemical structures of emodin and its derivatives and their inhibitory activities on tyrosine phosphorylation of *HER-2/neu* and proliferation in MDA-MB-453 cells

	Inhibitory activity (IC_{50}, in μM)[a]	
	Tyrosine phosphorylation of HER-2/neu[b]	*Proliferation of cells*[c]
Group A		
Emodin	21	5 + 0.4
DK-III-8	> 100	81 + 6.2
DK-III-19	> 100	45 + 3.6
DK-III-13	100	60 + 5.4
DK-III-11	> 100	> 100
Group B		
DK-II-1	> 100	> 100
DK-II-2	> 100	100 + 9.6
Group C		
DK-III-52	> 100	> 100
Group D		
DK-V-47	17	1 + 0.1
DK-V-48	> 100	> 100

[a]Concentration of reagents needed for 50% growth inhibition of cells. [b]Cells were treated by different concentrations of emodin and derivatives in the absence of serum for 24 h, then tyrosine phosphorylation of *HER-2/neu* were analysed by Western blot and quantitated by 'NIH Image' software program as described in Materials and methods. [c]Cells were treated by different concentrations of emodin and derivatives for 72 h in the presence of serum, then proliferation of cells were measured by MTT assay as described in Materials and methods

colonization assay, the colony formation activity of both breast cancer cells MDA-MB-453 and MCF-7 were suppressed in a dose-dependent manner. However, at the same concentrations of the drug (either at 0.5 or at 2.0 μM) the *HER-2/neu* overexpressing MDA-MD-453 cells (Figure 2a) are much more sensitive to the drugs than the MCF-7 cells (Figure 2b) which express basal levels of $p185^{neu}$ (at 0.5 μM, $P<0.001$; at 2.0 μM, $P<0.001$).

In addition, as shown in Figure 2, the activity of DK-V-47 to suppress anchorage-independent growth is much more potent than that of emodin ($P<0.0001$), for example, at a concentration of 0.5 μM, DK-V-47 shows significant inhibitory activity for the *HER-2/neu*-overexpressing MDA-MB-453 cells in colony formation, to achieve similar inhibition, higher than 20 μM emodin is required. The profound effect of the differential suppression of DK-V-47 and emodin on MDA-MB-453 cells in the soft agarose colonization assay compared with other biological assays such as the tyrosine phosphorylation of $p185^{neu}$ and the cell proliferation by MTT assay, as shown in Table 1, could be due to the fact that the different incubation times of the compounds with cells are required for different assays; 2–3 weeks are needed for the soft agarose colonization assay, whereas only 2–3 days are needed for other assays.

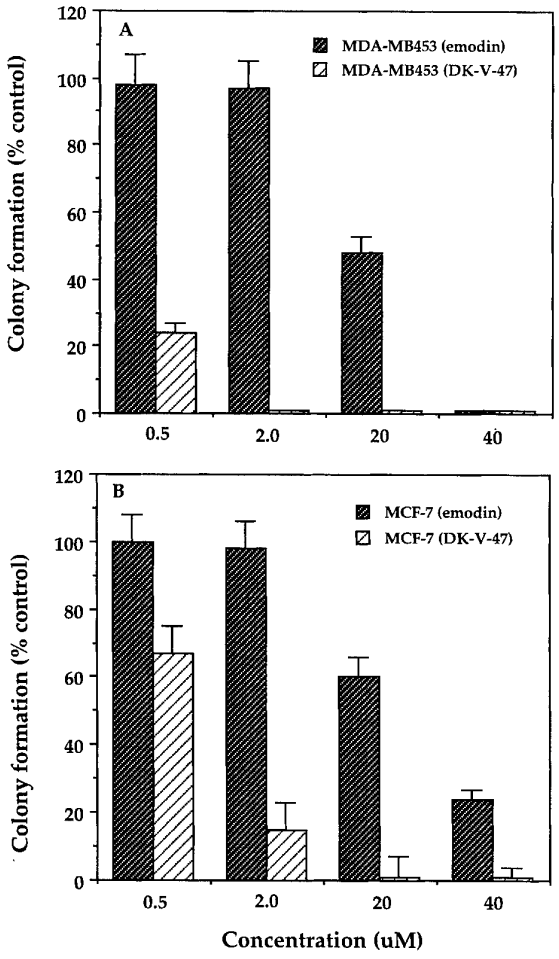

Figure 2 Emodin and DK-V-47 repress colony formation of human breast cancer cells in soft agarose. MDA-MB-453 cells (**a**) and MCF-7 cells (**b**) were seeded into 24-well plates (1×10^3 cells/well), in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without different concentrations of emodin or DK-V-47 incubated for 3 weeks at 37°C. Colonies were stained with *p*-iodonitro-tetrazolium violet and counted. The percentage of colony formation was calculated by defining the number of colonies in the absence of either emodin or DK-V-47 as 100%. All measurements were made four times; the results are means + s.d.

Emodin and DK-V-47 induce G_0/G_1 arrest

Since cell cycle distribution is closely related to cell proliferation rate, both effective compounds, emodin and DK-V-47 were further subjected to cell cycle distribution analysis (Figure 3). The number of cells in S-phase is apparently reduced and those in G_0/G_1 phase significantly increased. The results indicated that both compounds are able to inhibit DNA replication and to induce cell cycle arrest at G_0/G_1 phase, suggesting that cell cycle arrest may contribute to the growth inhibition.

Cell growth inhibition by emodin is reversible

In order to determine if inhibition of emodin on cell growth is stable or transitory, we treated MDA-MB-453 cells with 40 μM emodin for 3 days in the presence of serum, and washed off the reagents. These cells were then incubated with fresh serum-containing medium along with or without emodin for different time intervals. The viable cells was then measured by MTT assay and the fraction of viable cells was calculated by defining the absorption of cells without treatment of emodin as 100%. As shown in Figure 4, after cells were treated with 40 μM of emodin for 3 days, the fraction of viable cells decreased to 38% compared with untreated cells. For the cells continuously treated with emodin for the additional 5 days, the cell growth remained inhibited. However, when emodin was washed out after 3-day treatment, the cells started to grow and the growth rate was similar to the untreated cells in the subsequent time course. These results indicate that inhibitory effect by emodin is reversible.

Repression of autophosphorylation and transphosphorylation by emodin and DK-V-47 in vitro

While the results just described suggest that tyrosine kinase inhibitors such as emodin and DK-V-47 preferentially suppress the transformation phenotype of *HER-2/neu*-overexpressing MDA-MB-453 human cancer cells, it should be noted that these human cancer cells are derived from different patients and are likely associated with multiple genetic alterations. *HER-2/neu* expression may not be the only genetic difference between these two cancer cell lines.

To specifically investigate the effects of the tyrosine kinase inhibitors on *HER-2/neu* mediated transformation phenotypes, we would need transformed cells of which the transformation phenotypes are induced solely by *HER-2/neu* oncogene. To achieve this, we used an NIH3T3 transfectant, B104-1-1, which was transformed by the transfection of the mutation-activated rat *HER-2/neu* oncogene (Hung *et al*., 1989;

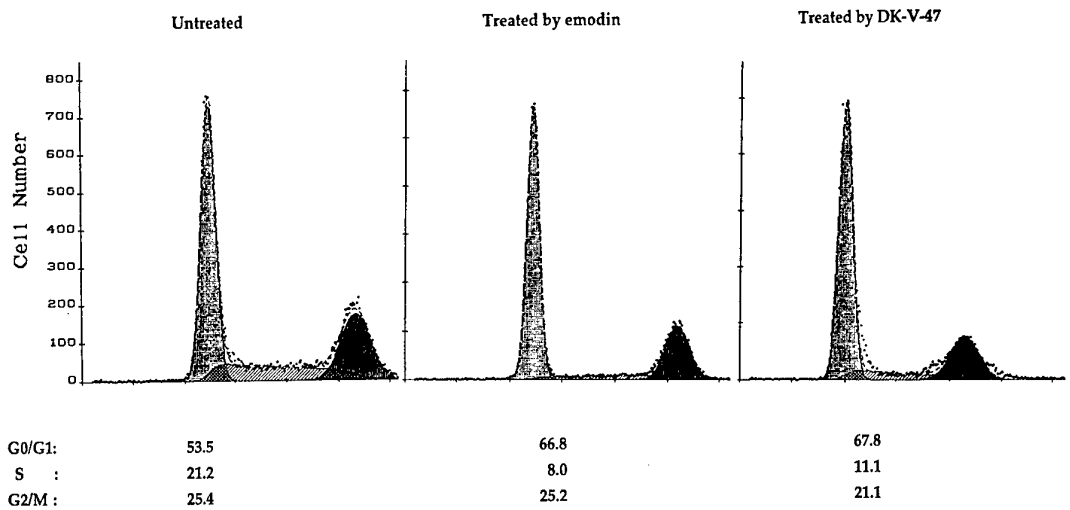

Figure 3 Emodin and DK-V-47 induce G_0/G_1 arrest. MDA-MB 453 cells were treated with or without either emodin (40 μM) or DK-V-47 (20 μM) for 3 days and trypsinized, the DNA content analyses were analysed as described under Materials and methods

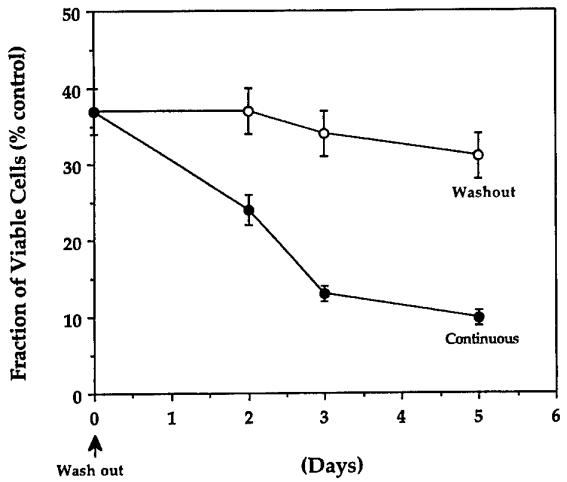

Figure 4 Cell growth inhibition by emodin is reversible. MDA-MB 453 cells were treated with or without 40 μM emodin in the presence of serum for 3 days, and then washed off the reagents. After that, the cells were incubated with fresh medium either in the presence or absence of emodin for different times, the viable cells were measured by MTT assay, and fraction of viable cells was calculated by defining the absorption of cells without treatment of emodin as 100%. All determinations were made in triplicate

Bargmann *et al*., 1986; Bargmann and Weinberg, 1988). The mutation-activated $p185^{neu}$ in B104-1-1 is known to be associated with high tyrosine kinase activity. The transformation phenotypes associated with B104-1-1 cells are induced solely by the *HER-2/neu* oncogene. Therefore, the B104-1-1 cells served as an excellent model system to study the effects of emodin and DK-V-47 on the transformation phenotype induced by *HER-2/neu* oncogene. In addition, the phenotype induced by expression of mutated $p185^{neu}$ has been reported to be similar to overexpression of normal $p185^{neu}$ that also results in overall increase of tyrosine kinase activity. Therefore, the mutated $p185^{neu}$ has been used as a model to understand overexpression of normal $p185^{neu}$.

To address whether emodin and DK-V-47 can directly inhibit the tyrosine kinase activity of activated *HER-2/neu*, $p185^{neu}$ was immunoprecipitated from the untreated B104-1-1 cells. The precipitates were then treated with varying concentrations of emodin and DK-V-47, and the kinase activity was measured, as shown in Figure 5, the tyrosine kinase activity for both autophosphorylation for $p185^{neu}$ and transphosphorylation for enolase is inhibited by both emodin and DK-V-47 in a dose-dependent manner. DK-V-47 is more potent than emodin, a finding which is consistent with tyrosine phosphorylation status of $p185^{neu}$ in breast cancer cells shown in Table 1. These results further demonstrate that both emodin and DK-V-47 directly inhibit the tyrosine kinase activity of $p185^{neu}$ complex.

DK-V-47 is more potent than emodin in inhibiting both anchorage-dependent and -independent growth of B104-1-1 cells

Next, we examined the effects of the two tyrosine kinase inhibitors on cell growth rate. As shown in Figure 6a, the growth of *HER-2/neu* transformed 3T3 cells, B104-1-1 cells was inhibited by both emodin and DK-V-47 to varying degrees in a dose-dependent manner. At 80 μM concentration, for example, emodin and DK-V-47 blocked 55% and 83% of the growth of B104-1-1 cells respectively. However, under the same condition, both emodin and DK-V-47 had little effect on the parental 3T3 cells.

These results indicate that emodin and DK-V-47 preferentially suppress growth of *HER-2/neu* transformed cells, and that DK-V-47 is more potent than emodin. They also suggest that the differential suppression effect occurs through the inhibition of the $p185^{neu}$ tyrosine kinase activity, because the tyrosine kinase inhibitors have virtually no effect on the parental 3T3 cells. When the anchorage-independent growth activity of B104-1-1 cells was measured by a soft agarose colonization assay as shown in Figure 6b,

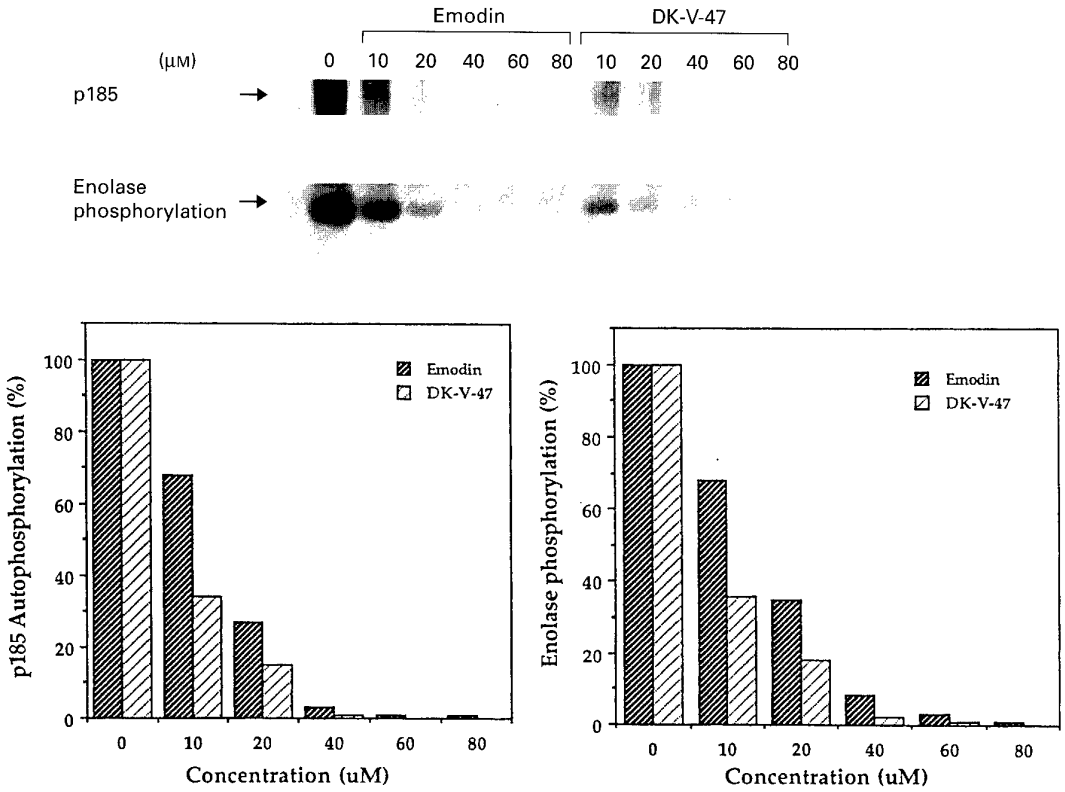

Figure 5 Emodin and DK-V-47 inhibit both autophosphorylation and transphosphorylation of p185^{neu} in activated *HER-2/neu* transformed 3T3 cells. Cell lysates from untreated cells were immunoprecipitated, then incubated with [γ-^{32}P]ATP, enolase and different concentrations of emodin and DK-V-47 at room temperature for 20 min. Reactants were resolved on 7.5% SDS–PAGE. The phosphorylation products were dried and visualized by autoradiography, as described in Materials and methods

DK-V-47 again behaved much more effectively in suppressing activity than did emodin.

Emodin and DK-V-47 suppress metastasis-associated properties induced by HER-2/neu *oncogene*

Tumor metastasis is a complex process involving a sequential series of critical steps (Liotta, 1986; Nicolson, 1988, 1991). The impartation of tumor cells in microcirculation and the subsequent invasion of blood vessel basement membrane are very important steps during blood-born metastasis. Gelatinase, type IV collagenases, has been shown to play a critical role in the dissolution of the basement membrane collagen during tumor cell invasion and metastasis (Nicolson, 1989). We have previously demonstrated that *HER-2/neu* transformed 3T3 cells can induce experimental metastasis in nude mice and enhance gelatinase activities (Yu and Hung, 1991). To determine whether or not emodin and DK-V-47 can decrease the activity of gelatinase collagenase IV in B104-1-1 cells, we examined the gelatinase activity in B104-1-1 cells with zymographic analysis. As shown in Figure 7, both DK-V-47 (A) and emodin (B) inhibit gelatinolytic activity of the 92 kDa and 68 kDa gelatinase, and this inhibition is enhanced with higher concentrations of both compounds. These results demonstrate that DK-V-47 is more effective than emodin; for example, at a concentration of 10 μM, DK-V-47 shows significant inhibitory activity for gelatinase, to achieve similar suppression, 40 μM of emodin is required.

Cancer cell invasion is very important; to be metastatic, malignant cells in the blood must extravasate from the circulation, invade basement membrane and colonize distant sites. Activated *HER-2/neu* transformed 3T3 cells have been shown to be invasive (Yu and Hung, 1991). To determine whether or not emodin and DK-V-47 can abolish the invasive properties of activated *HER-2/neu* transformed cells, we performed *in vitro* invasion assay to monitor their effects. As shown in the Figure 8, both compounds can virtually abolish the ability of activated *HER-2/neu* transformed cells to penetrate the Matrigel layer. For repressing the invasive ability of *HER-2/neu* transformed cells, DK-V-47 is again more effective than emodin ($P<0.0001$ at 25 μM and $P<0.05$ at 50 μM).

Using the NIH3T3 cells transformed by the mutation-activated *HER-2/neu* oncogene, B104-1-1, as a model system, the results shown in Figures 5–8 clearly demonstrate that both emodin and DK-V-47 can inhibit the tyrosine kinase activity of p185^{neu} and thus abolish the transforming phenotypes induced by the *HER-2/neu* oncogene. These results support the conclusion that repression of p185^{neu} tyrosine kinase by these two compounds is probably the major mechanism which induces the preferential suppression of

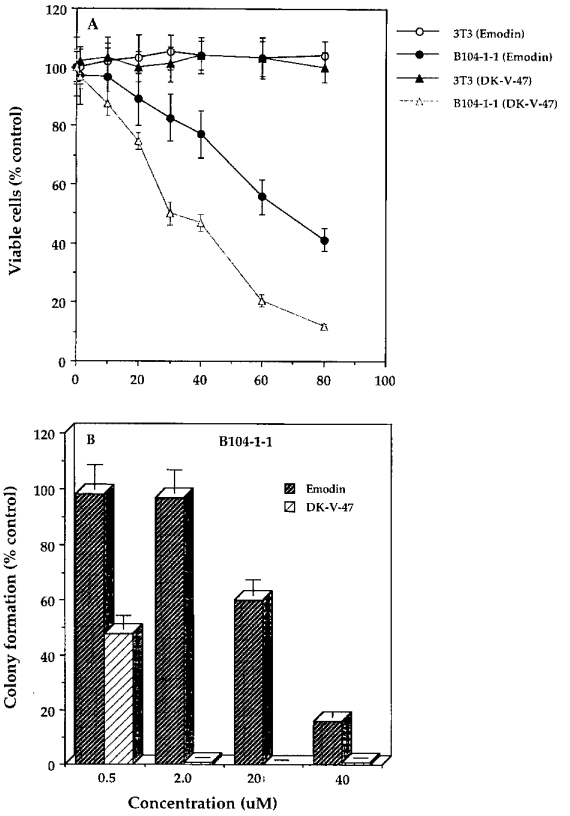

Figure 6 Emodin and DK-V-47 repress both anchorage-dependent and -independent growth of activated *HER-2/neu* transformed 3T3 cells. (**a**) Cells were incubated in the presence of serum along with or without different concentrations of emodin or DK-V-47 at 37°C for 72 h. The effect on cell growth was examined by an MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin and DK-V-47, the controls, as 100%. All determinations were made three times; the results are means + s.d. (**b**) A soft agarose assay was performed as described in the Figure 2

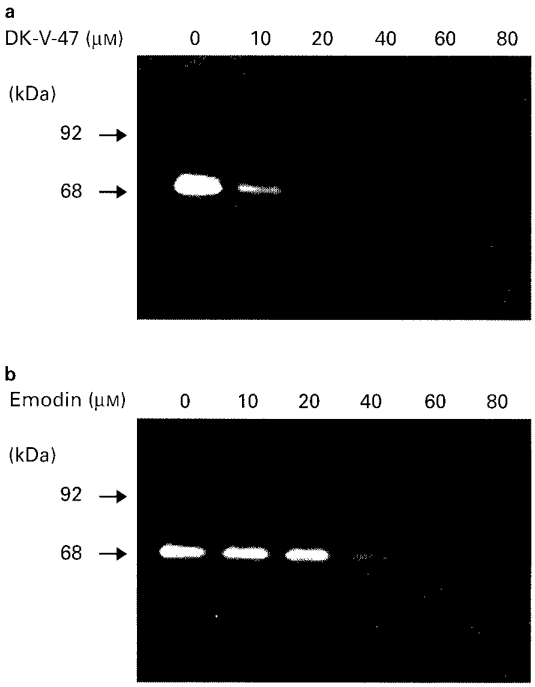

Figure 7 Emodin and DK-V-47 reduce gelatinolytic activity of activated *HER-2/neu*-transformed 3T3 cells. Cells were treated or not treated overnight with different concentrations of DK-V-47 (**a**) and emodin (**b**). The culture supernatants were then collected and analysed with zymography using gelatin-embedded SDS–PAGE. Gelatinolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained gels, as shown by the positions of both the 92 kDa and the 68 kDa gelatinase

biological activities for *HER-2/neu*-overexpressing cancer cells. The results also indicate that DK-V-47 is a much stronger suppressor than is emodin for *HER-2/neu*-induced transformation phenotypes.

Discussion

In our previous study (Zhang *et al*., 1995), we demonstrated that emodin suppresses the tyrosine kinase activity of *HER-2/neu* and preferentially inhibits cell growth and the transformation phenotype *in vitro* for human breast cancer cells that overexpress the *HER-2/neu* oncogene. Furthermore, we found that emodin can sensitize *HER-2/neu* overexpressing non-small-cell lung cancer (NSCLC) cells to chemotherapeutic drugs such as cisplatin, doxorubicin and VP16, to which these cells are resistant (Zhang and Hung, 1996).

In the present study, we examined the relationship between the chemical structure and the inhibitory activity of emodin and its nine derivatives on the tyrosine phosphorylation of $p185^{neu}$ and on the proliferation of human breast cancer cells. We identified that one of the nine derivatives, DK-V-47, is more effective than emodin in repressing the tyrosine phosphorylation of $p185^{neu}$ and in suppressing the transformation phenotype induced by the *HER-2/neu* oncogene. We have also found that emodin and DK-V-47 may arrest cell growth at G_0/G_1 phase by increasing numbers of cells in G_0/G_1 phase and reducing the numbers of cells in S-phase, under the same condition, we did not observe any apoptosis induced by treatment of either emodin or DK-V-47 (data not shown).

In our previous studies, we used human breast and lung cancer cells with different levels of *HER-2/neu* expression. It is well known that human cancer cell lines are associated with multiple genetic alterations, and *HER-2/neu* expression is not the only difference among the various human cancer cell lines. To investigate the specific effects of the tyrosine kinase inhibitors on the tyrosine kinase of $p185^{neu}$, in the present study, we used a *HER-2/neu*-transformed NIH3T3 cells line, B104-1-1, as a model. The results clearly indicate that DK-V-47 is a more potent suppressor than emodin in repressing the tyrosine kinase activity of $p185^{neu}$ (Figure 5) and, accordingly, in transformation phenotypes induced by the *HER-2/neu* oncogene. In addition to differential activity to repress tyrosine kinase of $p185^{neu}$, we could not rule out the possibility that membrane permeability might also contribute to the different potencies between emodin and DK-V-47. Taken together, this study provides strong evidence that repression of the $p185^{neu}$ tyrosine kinase activity by emodin and DK-V-47 plays a major

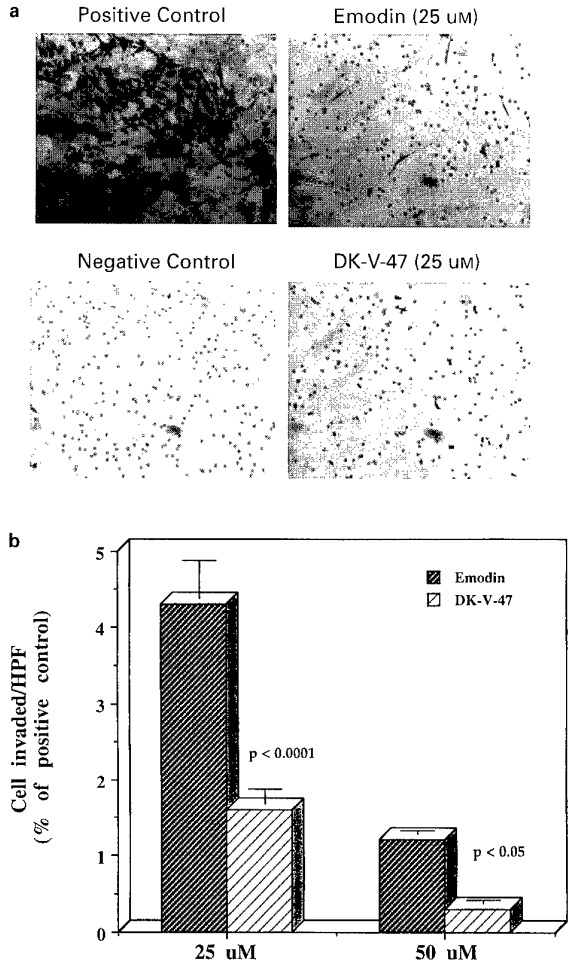

Figure 8 Emodin and DK-V-47 inhibit *in vitro* invasion of activated *HER-2/neu*-transformed 3T3 cells. *In vitro* invasion was measured by using 24-well Transwell units with an 8 mm pore polycarbonate filter coated with Matrigel to form a thin, continuous layer on the filter top. The lower compartment contained 0.6 ml of laminin (20 mg/ml) as a chemo-attractant. Activated *HER-2/neu*-transformed cells (5×10^4) were placed in the upper compartment and treated or not treated for three days with emodin or DK-V-47 in the presence of serum. The lower surfaces of the filters from the Transwell units were then fixed with 3% glutaraldehyde in PBS and stained with Giemsa solution (**a**). Chemo-invasive activity was determined under the microscope by counting cells that had migrated to the lower side of the filter (**b**). All experiments were done in triplicate; the results are means + s.d.

role in the preferential suppression effects of the *HER-2/neu*-overexpressing human cancer cells over the *HER-2/neu* basal level cancer cells.

With regard to the relationship between the structure of emodin and its nine derivatives and their inhibitory activity, the ten compounds were studied in four functional groups as shown in Table 1 and Figure 1. The first group is comprised of the compounds in which the CH_3 group at the C_3 position of emodin is replaced by one of four other functional groups. Their activities for the inhibition of the tyrosine phosphorylation of $p185^{neu}$ and the proliferation of cells are dramatically reduced; suggesting that the CH_3 group at the C_3 position is critical for these activities. The OH group at C_6 position of emodin is replaced by either H or OCH_3 group in the second group. These modifications also diminish the inhibitory activity as compared with emodin; this finding indicates that the OH group at the C_6 position is also critical for inhibitory activity. In the third group, the ketone group at C_{10} position of emodin is replaced by H group. Its inhibitory activities were significantly decreased as compared with emodin. These results indicate that the carbonyl group at C_{10} position is important for maintaining the inhibitory activity of emodin. The fourth group is comprised of the compounds in which the ketone at C_{10} position of emodin is replaced by the *p*-acetamidobenzylidene group. The compound DK-V-47 demonstrates more potent activity than does emodin. The CH_3CO group in the backbone of DK-V-47 appear to be critical for the biological activity; replacement of this group by H almost completely abolishes the biological activities. The hydroxyl group at C_6, methyl group at C_3 and the ketone group at C_{10} are important for the biological activity of emodin as shown in Table 1. Interestingly, DK-V-47 which does not have C_6 hydroxyl and C_3 methyl groups is also associated with profound potency. The data suggest that multiple functional groups may contribute to the biological activities of emodin backbone, and a more systematic study is required to address the detailed structural and functional relationship.

Scholar and Toews (1994) reported that the tyrosine kinase inhibitor genistein can inhibit the invasion of murine mammary carcinoma cells; repression of tyrosine phosphorylation may be the mechanism. We have previously shown that both the mutation-activated *HER-2/neu* oncogene and overexpression of the normal *HER-2/neu* gene can enhance critical metastatic potential including the invasive ability to penetrate the basement membrane preparation Matrigel (Yu and Hung, 1991; Yu *et al*., 1994). Both mutation-activation and the overexpression of *HER-2/neu* result in increased tyrosine kinase activity. And as shown in Figures 5, 7 and 8, we have shown that both emodin and DK-V-47 can inhibit the tyrosine kinase activity of mutation-activated *HER-2/neu* in 3T3 cells, and both compounds can repress the secretion of gelatinase and invasive potential. These findings suggest that tyrosine kinase activity of *HER-2/neu* is required for the metastatic process, and that inhibitors of the tyrosine kinase activity of *HER-2/neu* such as emodin and DK-V-47 can repress metastasis induced by activated *HER-2/neu*.

In our preliminary study, we found that emodin can also repress EGF-induced tyrosine phosphorylation of EGF receptor at higher concentration, compared with the concentration which used to repress tyrosine phosphorylation of *HER-2/neu* (data not shown). It would be interesting to further study whether emodin or DK-V-47 might have differential selectivity in repression of activities of different tyrosine kinase molecules.

In summary, our results indicate that a tyrosine kinase inhibitor such as emodin or DK-V-47 which is capable of repressing the tyrosine kinase activity of *HER-2/neu* can effectively inhibit the transformation, and *in vitro* invasion of *HER-2/neu* overexpressing cancer cells. The stronger tyrosine kinase inhibitor DK-V-47 may serve as a better suppressor of *HER-2/neu*-mediated transformation than emodin. These results may have important chemotherapeutic implications.

Materials and methods

Preparation of emodin derivatives

All emodin derivatives were prepared from emodin using the synthetic procedures described previously (Kim *et al.*, in press). These compounds are stable under the cell culture condition, no decomposition was observed within 72 h at 37°C.

Cell culture

Human breast cancer cells MDA-MB453, overexpressing *HER-2/neu* and MCF-7 cells expressing basal level of *HER-2/neu* were obtained from the American Type Culture Collection (Rockville, MD). The activated *HER-2/neu* transformed 3T3 cell line, B104-1-1, was established by transfecting the rat point mutation neu which possess high tyrosine kinase activity, into NIH3T3 cells (Hung *et al.*, 1989; Stern *et al.*, 1986; Bargmann and Weinberg, 1988). All cells were grown in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). Cells were grown in a humidified incubator at 37°C under 5% CO_2 in the air.

Western blot analysis

Cells were treated by different concentrations of emodin and derivatives for 24 h in the absence of serum, then cells were washed three times with PBS and lysed in lysis buffer (20 mM Na_2PO_4, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethysulfonyl fluoride, 10 mg/ml leupeptin, 100 mM NaF, 2 mM Na_3VO_4) as previously described (Zhang *et al.*, 1995). The protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio Rad Laboratories, Hercules, CA). A total of 50 μg of protein was separated by 6% SDS–PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing non-fat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). The treated filter paper was incubated with primary antibodies (anti-p185neu antibody c-*neu* [Ab-3] for the detection of $p185^{neu}$ or with anti-phosphotyrosine antibody [UBI, Lake Placid, NY] for the detection of phosphotyrosine). This was followed by incubation with HRP-goat-anti-mouse antibody (1 : 1000 dilution) (Boehringer Mannheim Corp., Indianapolis, IN). Bands were then visualized with an enhanced chemiluminescense system (Amersham Corp., Arangton, IL) and quantitated using 'NIH Image' software.

Immuno-complex kinase assay

The immuno-complex kinase assay was modified from those described previously (Zhang *et al.*, 1995). Briefly, untreated cells were collected and then lysed in lysis buffer. Cell lysates (500 μg) were incubated with monoclonal anti-$p185^{neu}$ antibody c-*neu* (Ab-3) for 1 h at 4°C, then precipitated with 50 μl of protein-A-conjugated agarose (Boehringer Mannheim) for 30 min at 4°C. The cells were then washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in an assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnC12). To 40 μl of beads (protein-a-conjugated agarose), 10 μCi of [γ-^{32}P]ATP (Amersham) and 10 μl of enolase (Sigma Chemical Co., St. Louis, MO) and 1.0 μl of different concentrations of emodin and DK-V-47 were added and incubated at room temperature for 20 min. The reactants were separated by 7.5% SDS–PAGE. The gel was then dried, visualized by autoradiography and quantitated by 'NIH Image' software.

Cell proliferation by MTT assay

Cells were detached by trypsinization, seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight, and then treated with different concentrations of test samples of emodin and its derivatives and incubated for an additional 72 h in the presence of serum. The effects on cell growth were examined by MTT assay (Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990). Briefly, 20 μl of MTT solution (5 mg/ml) (Sigma) was added to each well and incubated at 37°C for 4 h. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 ml of DMSO, and then monitored by a microplate reader (Dynatech MR 5000 fluorescence, Dynatech Corp., Burlington, MA) at a wavelength of 590 nM.

DNA content analysis

MDA-MB453 cells were treated with or without either emodin (40 μM) or DK-V-47 (20 μM) for 3 days in the presence of serum, then cells were collected by trypsinization and fixed for 30 min at −20°C in 70% ethanol, 30% phosphate buffered saline (PBS) mixture. After staining with PBS containing 50 mg/ml propidium iodide and 8 μg/ml RNase A, cells were analysed on EPICS PROFILE flow cytometer (Coulter) as described (Kiyokawa *et al.*, 1997).

Colony formation in soft agarose

Cells were seeded in 24-well plates (1×10^3 cells/well) in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer and incubated for 4 weeks at 37°C, as previously described (Zhang *et al.*, 1995). Colonies were then stained with p-iodonitrotetrazolium violet (1 mg/ml), and colonies larger than 100 μM were counted. Each measurement was made four times.

Zymography of gelatinolytic activity

As previously described (Yu and Hung, 1991; Yu *et al.*, 1994), cells were detached by trypsinization, seeded at 2×10^6 cells/well in a 6-well plate and cultured in DMEM/F12 medium supplemented with 1% FBS overnight, the cells were then washed with PBS and serum-free DMEM/F12 medium was added; then the cells were treated with different concentrations of test samples and incubated for an additional 24 h. The culture supernatants were collected, and then centrifuged at 800 *g* for 10 min, and then again at 18 000 *g* for 10 min. The supernatants (150 ml) were analysed by zymography using SDS–PAGE containing 1.5% gelatin prepared according to procedures described previously (Yu and Hung, 1991; Yu *et al.*, 1994).

In vitro *chemoinvasion assay*

In vitro invasiveness was conducted according to the procedure described previously (Yu and Hung, 1991; Yu *et al.*, 1994), with modifications. Briefly, 24-well Transwell units with 8-mm pore size polycarbonate filter (Costar Corp., Cambridge, MA) were coated with 0.1 ml of a 1 : 30 dilution (48 mg/filter) of Matrigel in cold DMEM/F12 medium. These filters were then air dried at room temperature, thus forming a continuous thin layer on top of the filter. The lower compartment contained 0.6 ml laminin (20 mg/ml, Becton Dickinson) as a chemoattractant or DMEM/F12 medium as a negative control. The cells (1×10^5 cells/0.1 ml of DMEM/F12 containing 0.1% bovine serum albumin) were placed in the upper compartment and incubated with or without either emodin or DK-V-47 at 37°C for 72 h in a humidified atmosphere of 95% air, and 5% CO_2. Following incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with Giemsa. The number of cells per high-power (×200) field

that had migrated to the lower side of the filter were counted.

Statistical analysis

Statistical analysis was performed with student's *t* test.

Abbreviations

Abbreviations used are: DK-V-47, 10-(4-acetamidobenzylidene)-9-anthrone; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; IP, immunoprecipitation; WB, Western blotting; PY, phosphotyrosine; ATP, adenosine triphosphate.

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Involvement of cdc2-mediated phosphorylation in the cell cycle-dependent regulation of p185^{neu}

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We previously reported cell cycle-dependent negative regulation of p185^{neu} (decreased tyrosine phosphorylation and kinase activity, with electrophoretic mobility retarded by serine/threonine phosphorylation) in M phase and the escape of mutation-activated p185^{neu*} from this regulation. Our present results showed that retardation of electrophoretic mobility occurs independently of the cells' transformed status. We found that normal p185^{neu} lost its ability to dimerize in the M phase. We demonstrated a physical association between cdc2 (a serine/threonine kinase, active in M phase) and p185^{neu}. We showed that the carboxy terminal portion of p185^{neu} is phosphorylated *in vitro* by cdc2. Many phosphopeptides (at least three phosphoserine residues) unique to the M phase were identified, and the *in vivo* and *in vitro* phosphopeptide patterns were superimposable. In contrast, mutation-activated p185^{neu*} dimerized in the M phase with no changes in electrophoretic mobility, failed to associate with cdc2 and no unique phosphoserine residues could be identified in the M phase (data not shown), consistent with the escape of p185^{neu*} from cell cycle-dependent regulation. Our results suggest that this escape is an intrinsic property of the mutation-activated p185^{neu*} independent of its ability to transform cells. Our results also suggest the involvement of serine/threonine kinases such as cdc2 in the cell cycle-dependent negative regulation of p185^{neu}.

Keywords: cdc2; cell cycle; p185^{neu}; phosphoamino acid analysis; peptide mapping; serine/threonine kinases

Introduction

The HER2/*neu*/c-*erb*B-2 gene encodes a 185 kDa transmembrane glycoprotein (p185^{neu}) that belongs to the EGF receptor family (Bargmann *et al.*, 1986a,b; Bargmann and Weinberg, 1988a,b; Hung *et al.*, 1986; Padhy *et al.*, 1982; Schechter *et al.*, 1984). Members of this family are characterized by an extracellular, transmembrane, and the cytoplasmic tyrosine kinase domains. While the extracellular domain is involved in ligand binding, the cytoplasmic carboxy terminal tails display sequence heterogeneity and carry several tyrosine (including the autophosphorylation sites), serine, and threonine residues (Yarden and Ullrich, 1988). In many cellular systems, both dimerization and tyrosine kinase activation follow second order kinetics with respect to receptor concentration, which implicates dimerization as an activating event for the kinase domain and suggests that the dimers are important structures in the propagation of mitogenic signals from the cell surface to the nucleus (Heldin, 1995).

It is known that a single-point mutation in the transmembrane region (Val-644 to Glu) of *neu* converts the *neu* protooncogene into a transforming *neu* oncogene (*neu**) (Bargmann *et al.*, 1986a; Bargmann and Weinberg, 1988b; Hung *et al.*, 1989; Muller *et al.*, 1988; Stern *et al.*, 1988). The mutation-activated p185^{neu*} is associated with higher tyrosine kinase activity and exhibits more potent transforming ability than normal p185^{neu} (Bargmann and Weinberg, 1988a; Stern *et al.*, 1988; Weiner *et al.*, 1989). Although a direct ligand of p185^{neu} is still not known, p185^{neu*} exists predominantly in the dimer form, and p185^{neu} exists as a monomer (Weiner *et al.*, 1989). Indeed, theoretical (Brandt-Rauf *et al.*, 1990; Smith and Bormann, 1996; Sternberg and Gullick, 1990) and experimental evidence (Weiner *et al.*, 1989) implied that the oncogenic mutation maintains the receptor in a dimer form that is analogous to the ligand-induced dimer of the EGF receptor (Yarden and Schlessinger, 1987a,b). By dimerizing the receptor, the oncogenic mutation seems to establish a high affinity binding state that is functionally equivalent to that of the ligand-occupied normal receptor (Ben-Levy *et al.*, 1992). We demonstrated that the structure and function of p185^{neu} are regulated through the cell cycle. In M phase, p185^{neu} is least active in tyrosine phosphorylation function and the regulation may occur through the alteration of phosphorylation of serine and/or threonine residues. Point mutation allowed p185^{neu} tyrosine kinase to escape cell cycle regulation as it remained constitutively active throughout the cell cycle (Kiyokawa *et al.*, 1995).

In the present study we sought further understanding of the mechanism of the cell cycle-dependent negative regulation of p185^{neu} we had shown. Using a cross-linking agent, we found that the normal p185^{neu} does not dimerize in the M phase, but that the oncogenic p185^{neu*} is dimerized. Further experiments with co-immunoprecipitation, *in vitro* kinase assays, and *in vivo* ^{32}P metabolic labeling methods, suggested the involvement of cdc2, an M phase-specific serine/threonine kinase, in the negative regulation.

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Results

Escape from cell cycle-dependent regulation is an intrinsic property of the mutation-activated p185neu, independent of its ability to transform cells*

As shown earlier, the normal p185neu protein is regulated in a cell cycle-dependent manner, whereas the p185neu* (with a point mutation in the transmembrane region) escapes from this regulation. With cell-cycle progression, tyrosine phosphorylation and tyrosine kinase activity of p185neu gradually decreased, reaching their minimum level in M phase. An overall hyperphosphorylation in the serine and/or threonine residues resulted in an electrophoretic mobility shift in the M phase. The oncogenic p185neu* protein tyrosine kinase was constitutively active in both G_0/G_1 and M phases while the protein's electrophoretic mobility did not change significantly. Thus, the single-point mutation in the transmembrane domain facilitated the escape of p185neu* from cell cycle dependent negative regulation of p185neu tyrosine kinase activity, possibly as a result of serine/threonine phosphorylation (Kiyokawa *et al.*, 1995). Since the single-point mutation also induced cellular transformation, it was pertinent to ask whether the absence of the mobility shift in M phase is a specific property of the mutated *neu** or is caused by the transformation *per se*. To address this question, NIH3T3 cells (expressing endogenous p185neu) transformed by different oncogenes (*ras*, *src* and *abl*) were synchronized either in G_0/G_1 or M phase and their electrophoretic mobility was analysed after their cell cycle stage was confirmed by DNA content analysis. As shown in the two upper panels of Figure 1, DNA content analysis confirmed that the serum starvation synchronized the parental NIH3T3 and the transfected cells in G_0/G_1 stage, and that nocodazole treatment arrested these cells in the M phase. The results, shown in the lower panel of Figure 1 depicting the electrophorectic mobility changes in p185neu detected by Western blotting, indicate retarded migration of the protein in M phase in all cells analysed. Mutated p185neu* did not show a change in electrophoretic mobility at different stages of the cell cycle (Kiyokawa *et al.*, 1995) and the cells transformed by various oncogenes (*ras*, *src* and *abl*) also showed retarded electrophoretic mobility (Figure 1). These results suggested that the escape from cell cycle-dependent regulation is an intrinsic property of the mutation-activated p185neu*, independent of its ability to transform cells.

*Normal p185neu fails to dimerize in M phase but not the mutation-activated p185neu**

Since the function of p185neu is tightly associated with its dimerization status (Weiner *et al.*, 1989), we next asked whether this could account for the negative regulation of p185neu in M phase and the escape of p185neu* from this process. We used derivatives of NIH3T3 cells that express the normal *neu* (DHFR/G8) and the mutated *neu** (B104-1-1). After synchronizing these cells in G_0/G_1 or M phase, they were crosslinked and p185neu proteins were analysed by Western blotting. As predicted, the DHFR/G8 cells failed to dimerize in the M phase, and the dimerization status remained unaltered in B104-1-1 cells between G_0/G_1 and M phases (Figure 2). Immunoprecipitation of p185neu after crosslinking under the same conditions also gave similar results (data now shown).

Added to our previous report (Kiyokawa *et al.*, 1995), these data suggested that, in M phase of the cell cycle, the serine/threonine hyperphosphorylation of p185neu may prevent receptor dimerization, which in turn inhibits the tyrosine kinase activity of p185neu and intrinsically aids the escape of p185neu* from this regulation.

Physical association between cdc2 and p185neu

Interestingly, cdc2, a serine/threonine kinase, is most active in M phase (Bischoff *et al.*, 1990; Draetta and Beach, 1988; Lee and Nurse, 1987; Shenoy *et al.*, 1989).

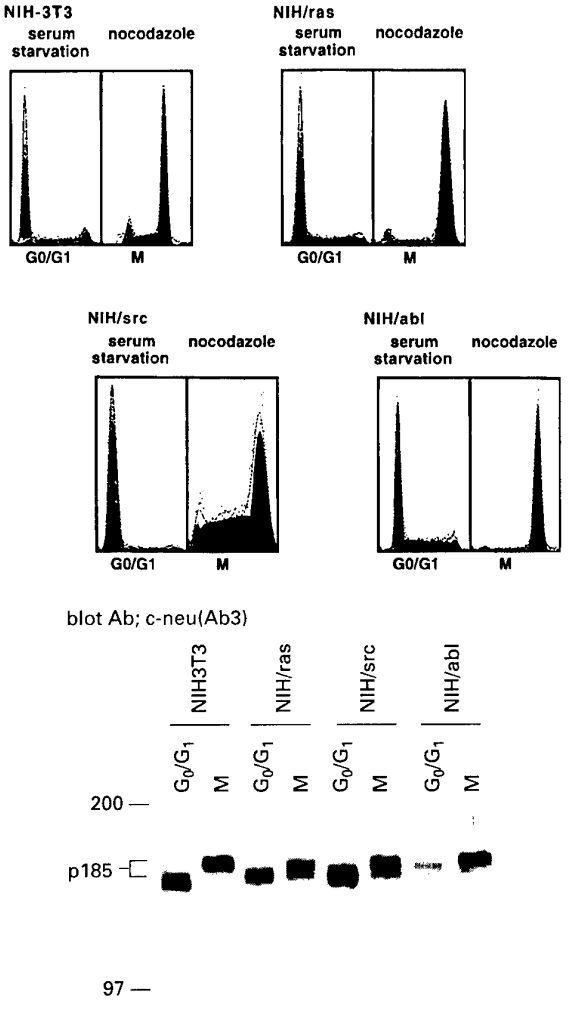

Figure 1 DNA content analysis and electrophoretic mobility changes in G_0/G_1 or M phase. Upper panel: NIH3T3, NIH/*ras*, NIH/*src*, and NIH/*abl* cells were either serum-starved (G_0/G_1) or treated nocodazole (M) and trypsinized for the DNA content analysis described in Materials and methods. Lower panel: Total lysates from either serum-starved (G_0/G_1) or nocodazole-treated (M) NIH3T3, NIH/*ras*, NIH/*src* and NIH/*abl* cells were analysed, after electrophoresis, by Western blotting with a monoclonal anti-p185neu antibody. The experiments were repeated at least twice with similar results

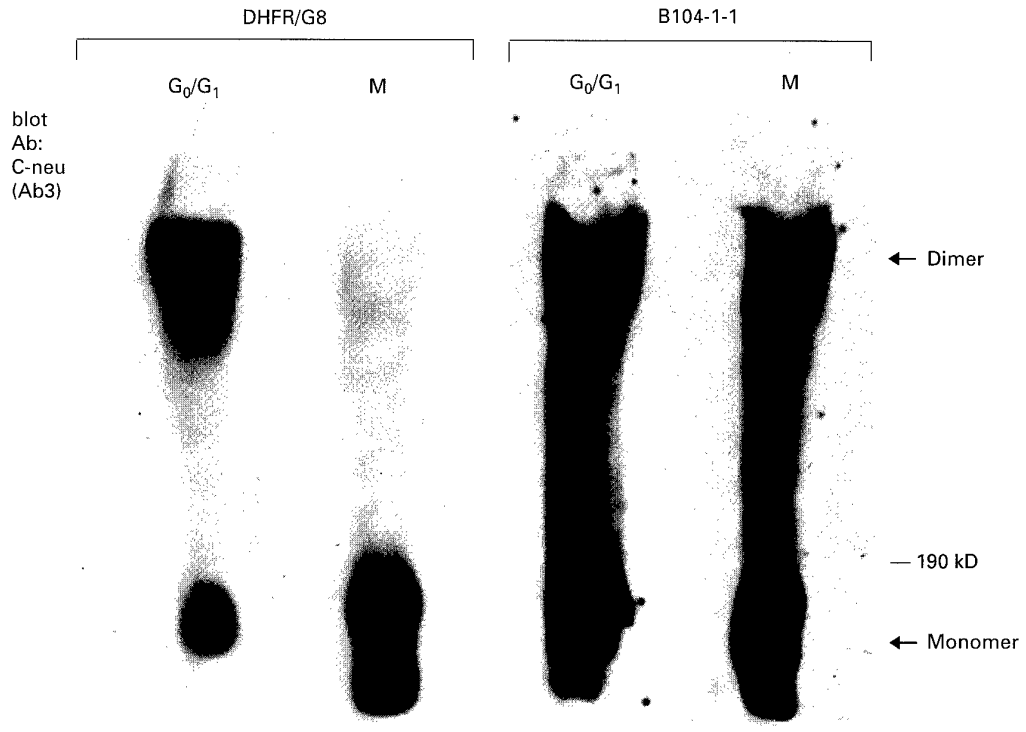

Figure 2 Dimerization in G_0/G_1 or M phase. DHFR/G8 and B104-1-1 cells were synchronized either in G_0/G_1 or M phase and then crosslinked with BS^3 as described in Materials and methods. After electrophoresis, the total lysates were analysed by Western blotting with a monoclonal anti-p185^{neu} antibody. The experiment was done twice with reproducible results

Perhaps not by coincidence, a careful examination of the amino acid sequence of p185^{neu} revealed no less than 17 potential sites for cdc2. To see whether the two different kinases, cdc2 and p185^{neu}, are physically associated, we immunoprecipitated the cell lysates of DHFR/G8 expressing normal p185^{neu} and B104-1-1 expressing p185^{neu*} with a monoclonal antibody to cdc2 or with normal mouse serum (NMS) and immunoblotted them with a monoclonal antibody to p185^{neu} or cdc2. The results shown in Figure 3a indicated that these two proteins are physically associated in DHFR/G8 cells, but not in B104-1-1 cells that express p185^{neu*}. These results were ascertained by using three different antibodies to immunoprecipitate cdc2 under the same experimental conditions (data not shown). We also examined the physical association between cdc2 and p185^{neu} in different stages of the cell cycle in DHFR/G8 cells, by chemical synchronization, and found relatively abundant association between them at G2/M phase (Figure 3b).

cdc2 can phosphorylate p185^{neu} in vitro

Encouraged by the physical association of cdc2 and p185^{neu} in DHFR/G8 cells, we used an *in vitro* complex kinase assay to investigate whether cdc2 can phosphorylate p185^{neu}. Phosphorylation of p185^{neu} by cdc2 was observed when the p185^{neu} was immunoprecipitated from the DHFR/G8 cells and then mixed with commercially available active cdc2, or when the cell lysates from DHFR/G8 cells were frozen and thawed a few times after immunoprecipitation (to reduce the autophosphorylation activity of p185^{neu} tyrosine kinase) and mixed (Figure 3c). Phosphorylation of p185^{neu} was not seen, however, when cdc2 or p185^{neu} was present alone in the reaction mixture (Figure 3c). The phosphorylated band of p185^{neu} from the above experiment was further examined by phosphoamino acid analysis, and the results confirmed the involvement of phosphorylation of serine and threonine but not of tyrosine residues (Figure 3d) which was predictable from the known action of cdc2 as a serine/threonine kinase. Using different antibodies to immunoprecipitate cdc2, we also confirmed that the immunoprecipitated p185^{neu} from DHFR/G8 cells could be phosphorylated by cdc2 *in vitro* (data not shown).

cdc2 phosphorylates the carboxy terminal of p185^{neu}

In an attempt to locate the phosphorylation sites of p185^{neu} in M phase by cdc2, we carried out an *in vitro* and *in vivo* two-dimensional phosphopeptide mapping analysis. The approximate positions of the potential phosphorylation sites for cdc2 in the p185^{neu} protein are schematically shown in Figure 4a. The drawing depicts GST-fusion protein that represents the carboxy terminal region of p185^{neu} (YX1) and contains most of the serine and threonine residues, and another GST protein (YX3) that contains a few serine and threonine residues constructed from the kinase domain and the juxtamembrane portion of p185^{neu}. The expression of these fusion proteins was revealed by coomassie blue staining of the bacterial cell lysates and the major

proteins of expected size were stained heavily by the dye and further confirmed by immunoblotting analysis, which showed that an antibody directed against the carboxy terminal tail of *neu* detected only YX1 and not YX3 (Figure 4b). Similarly, the use of an antibody against the kinase domain reacted with YX3 but not YX1, as expected (Figure 4b). These fusion proteins were then used in an *in vitro* complex kinase assay that utilized the commercially available purified cdc2 (UBI). The results showed that only YX1 made from the carboxy terminal of p185^{neu} was phosphorylated when mixed with cdc2, and that cdc2 : YX3 mix had no such effect (Figure 4c). Phosphorylation was not observed when either YX1 or YX3 was present in the reaction mixture without cdc2 (data not shown). Taken together, these results strongly suggested the existence of potential phosphorylation sites for cdc2 at the carboxy terminal tail of p185^{neu}.

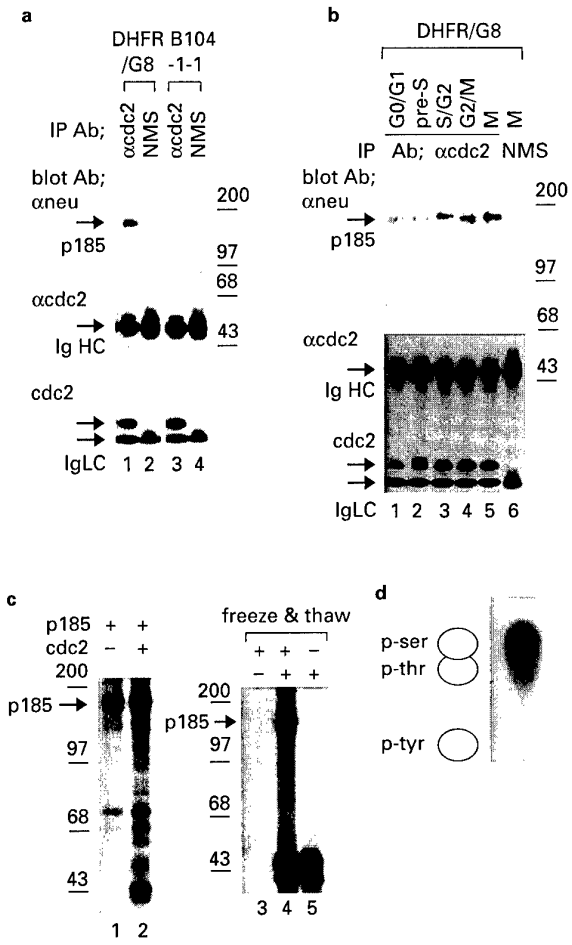

Figure 3 Physical association of normal p185^{neu} with, and its phosphorylation *in vitro* by, cdc2. (**a**) Co-immunoprecipitation of p185^{neu} with cdc2. The proteins were immunoprecipitated with a monoclonal anti-cdc2 antibody (α cdc2) or normal mouse serum (NMS) from 500 μg of precleared lysate of DHFR/G8 or B104-1-1 cells. After intensive washing with lysis buffer, samples were separated on 6–10% SDS–PAGE gradient gel and transferred onto a nitrocellulose membrane. The membrane was cut into two parts to detect both p185^{neu} and cdc2 by immunoblotting with either monoclonal anti-p185^{neu} antibody (c-*neu* (Ab3), upper panel) or monoclonal anti-cdc2 antibody (αcdc2, lower panel). (**b**) Cell cycle dependent association of p185^{neu} with cdc2. DHFR/G8 cells were synchronized at different stages of cell cycle as described in Materials and methods. Cell lysates were prepared, immunoprecipitated and analysed as described for a. (**c**) *In vitro* phosphorylation of p185^{neu} by cdc2. The ability to phosphorylate p185^{neu} was tested by an *in vitro* immune complex kinase assay. Immunoprecipitated p185^{neu} proteins from DHFR/G8 were equally divided into two parts and mixed with (+) or without (−) the purified cdc2 protein (25 ng/assay) followed by incubation with [γ-^{32}P]ATP and $MgCl_2$ for 20 min at room temperature. After separation on 7% SDS–PAGE gel samples were exposed on X-ray film. To minimize autophosphorylation activity, proteins denatured by repeated freezing and thawing were tested in a similar manner. (**d**) Phosphoamino acid analysis. Phosphorylated p185^{neu} proteins from lane 4 of **c** were excised and phosphoamino acid analysis was performed as described in Materials and methods. Ig HC-Immunoglobulin heavy chain; Ig LC-Immunoglobulin light chain. The results from **a**, **b**, **c** and **d** were confirmed at least in another independent experiment

In vitro *and* in vivo *phosphopeptide patterns are superimposable*

The possible relevance of cdc2-mediated phosphorylation of p185^{neu} observed *in vitro* was next examined *in vivo* by metabolic labeling of DHFR/G8 cells with ^{32}P before (unsynchronized) or after the cells were synchronized in G_0/G_1 or M phase and their phosphopeptide pattern analysed, as shown in Figure 5. When phosphopeptides from G_0/G_1 and M phase were mixed together and analysed, more than nine phosphopeptides unique to the M phase could be identified, along with two phosphopeptides unique to the G_0/G_1 phase, and several phosphopeptides common to both G_0/G_1 and M phases. Phosphoamino acid analysis of the phosphopeptides revealed that at least three phosphopeptides unique to the M phase contained phosphorylated serine residues, and several such residues were found also in the phosphopeptides common to both phases of the cell cycle (Figure 5). When a similar experiment was done with B104-1-1 cells, the phosphopeptide patterns of G_0/G_1 and M phases were virtually identical (notably, they resembled the G_0/G_1 stage of DHFR/G8 cells), and no unique phosphoserine residues could be identified in the M phase (data not shown), consistent with the escape of p185^{neu*} from cell cycle-dependent regulation. Thus, our results suggested that cdc2 can phosphorylate p185^{neu} in M phase in DHFR/G8 cells. If this is indeed the case, the *in vitro* and *in vivo* phosphopeptide mapping patterns should be similar. When the phosphopeptides obtained from the YX1-cdc2 complex (Figure 4c) were mixed with the *in vivo* -labeled phosphopeptides prepared from the M phase synchronized DHFR/G8 cells and analysed, we found more than 10 common phosphopeptide spots among the *in vivo* and *in vitro* patterns (Figure 6). Phosphoamino acid analysis helped us to identify at least five phosphoserine-containing phosphopeptides among them, supporting the involvement of cdc2. The presence of several such peptides unique to the M phase (Figure 6) indicated that other serine/threonine kinases could also be involved in the negative regulation of p185^{neu}.

Discussion

We pursued our earlier report which showed the disruption of the cell-cycle dependent regulation of normal p185^{neu} by its oncogenic counterpart p185^{neu*} and defined a plausible mechanism of cellular

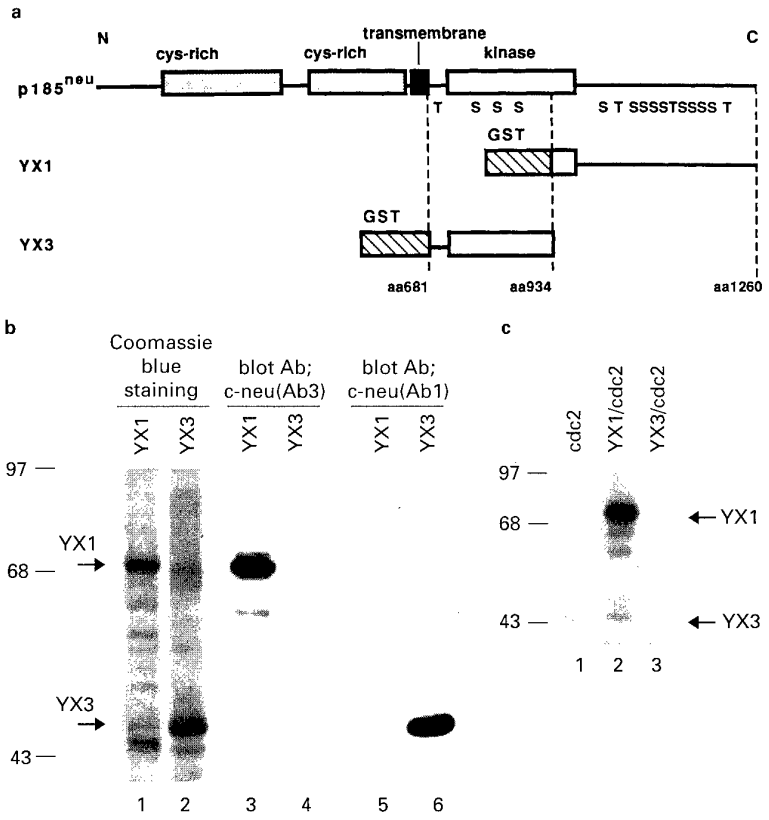

Figure 4 *In vitro* phosphorylation of recombinant p185^{neu} protein of cdc2. (**a**) Construction of GST-fusion p185^{neu} proteins are shown by the schema. (**b**) Recombinant-GST-fusion p185^{neu} proteins were purified as described in Materials and methods, and their molecular weights were examined by SDS–PAGE followed by coomassie blue staining (lanes 1 and 2). Both proteins (YX1 and YX3) were immunoblotted with either anti-p185^{neu} c-terminal antibody (c-*neu* Ab-3; lanes 3 and 4) or anti-p185^{neu} kinase domain antibody (c-*neu* Ab-1; lanes 5 and 6). (**c**) Purified cdc2 protein (25 ng/assay) was incubated with YX1 (lane 2) or YX3 (lane 3) or without the recombinant proteins (lane 1) in the presence of [γ-^{32}P]ATP and $MgCl_2$. The ability of cdc2 to phosphorylate the fusion proteins was examined as described in Figure 3. Similar results were obtained when the experiment was repeated two times

transformation (Kiyokawa *et al*., 1995). The present results clarified that the disruption is not caused by the transformation *per se* (Figure 1), but that the escape of p185^{neu*} from regulation may be an intrinsic property of the mutated protein. We had predicted that the negative regulation of normal p185^{neu} tyrosine kinase activity observed in M phase of the cell cycle in DHFR/G8 cells and an escape from this regulation in B104-1-1 cells that express p185^{neu*} (Kiyokawa *et al*., 1995) could be the result of a disequilibrium between the p185^{neu} receptor's monomeric and dimeric forms. The results shown in Figure 2 lend strong support to this prediction because the dimers could not be captured by crosslinking during M phase in DHFR/G8 cells. Weiner *et al*. (1989) have analysed the formation of dimers in detail in DHFR/G8 and B104-1-1 cells. They found that about 70% of the oncogenic p185^{neu*} exists as an aggregated cross-linkable complex, and 20–30% of normal p185^{neu} in DHFR/G8 cells can be cross-linked under identical conditions. It is interesting to note that, in a family of bacterial receptors that have the same topology as the eukaryotic single-transmembrane receptors, general structural changes (such as a monomer-to-dimer transition) seem to have been responsible for transmembrane signaling rather than specific ligand-induced conformational changes (Stock, 1996). Ligand-independent dimerization is known to occur in oncogenic tyrosine and serine/threonine receptor kinases (Adelsman *et al*., 1996; Carlberg and Rohrschneider, 1994; Vivien *et al*., 1995; Watowich *et al*., 1992; Weiner *et al*., 1989).

More intriguing is the mechanism by which such negative regulation results in the failure to dimerize (Figure 2) and the consequent decrease in tyrosine kinase activity and signaling of the normal p185^{neu} receptor (Kiyokawa *et al*., 1995) in M phase. We hypothesized that it is due to the hyperphosphorylation of serine/threonine residues in M phase and the amino acid mutation in p185^{neu*} disrupts this regulation. The presence of more phosphopeptide spots (at least 3 containing phosphoserine residues) in the M phase than in the G_0/G_1 phase (which had no phosphoserine residues) of DHFR/G8 cells that express normal p185^{neu} (Figure 5), and identical phosphopeptide patterns of G_0/G_1 and M phase-synchronized B104-1-1 cells that express mutated p185^{neu*} (data not shown), also support this possibility. The presence of 17 cdc2 consensus sites in the amino acid sequence of p185^{neu} and cdc2's role as a serine/threonine kinase most active in M phase (Bischoff *et al*., 1990; Draetta and Beach, 1988; Lee and Nurse, 1987; Shenoy *et al*., 1989)

qualifies it as a potential candidate for the described negative regulation. Consistent with these structural features and M phase-specific activity are our experimental results (Figure 3a and b) which show that physical association between these two proteins is stronger at G2/M phase of the cell cycle. Though cdc2 is a nuclear protein, its existence and activation in the cytoplasm has also been reported (Heald *et al*., 1993). Our results demonstrated cdc2 site of action to be the carboxy terminal of p185^{neu} (Figure 4) after showing cdc2 to be capable of phosphorylating p185^{neu} *in vitro* and the phosphorylation sites to overlap with those *in vivo* (Figures 3 and 5). Furthermore, the superimposable phosphopeptide patterns obtained when *in vitro* cdc2-phosphorylated YX1 (carboxy-terminal p185^{neu} -GST fusion protein) was mixed with the *in vivo* labeled M phase-synchronized DHFR/G8 cells (Figure 6) strongly suggested the involvement of cdc2. Further work is needed to locate specific serine/threonine residues in p185^{neu} phosphorylated by cdc2. Mutation of such residues would reveal their relative functional importance, though the question is complicated by the presence of several serine/threonine residues. Purified *Xenopus laevis* p34cdc2 was reported to phosphorylate the EGFR on serine 1002 *in vitro*, implying that receptor function may be regulated in a cell cycle-dependent fashion (Kuppuswamy *et al*., 1993). In addition, the *in vitro* phosphorylation was associated with an inhibition of EGFR tyrosine kinase activity. A comparison of the sequences of several growth factor receptors showed that the string of acidic residues, followed by the Ser-Pro motif found at residues 1002 1003 of the EGFR, a proposed cdc2 phosphorylation site, is conserved in most EGFR family members, including p185^{neu} (Bargmann *et al*., 1986a), ErbB-3 (Plowman *et al*., 1990), and let-23, the EGFR homologue in *C. elegans* (Aroian *et al*., 1990). However, a role for these cell cycle stage-specific phosphorylations remains an intriguing possibility in the negative regulation of the receptor tyrosine kinases, including the EGFR and p185^{neu}. A better known physiological feedback-inhibitory mechanism involves protein kinase C (PKC), a serine/threonine kinase, which phosphorylates threonine 654 residue on the EGFR, decreasing the receptor's binding affinity for ligands (Morrison *et al*., 1993), diminishing tyrosine kinase activity (Cochet *et al*., 1984; Davis, 1988), and attenuating signals from the EGFR (Chen *et al*., 1996).

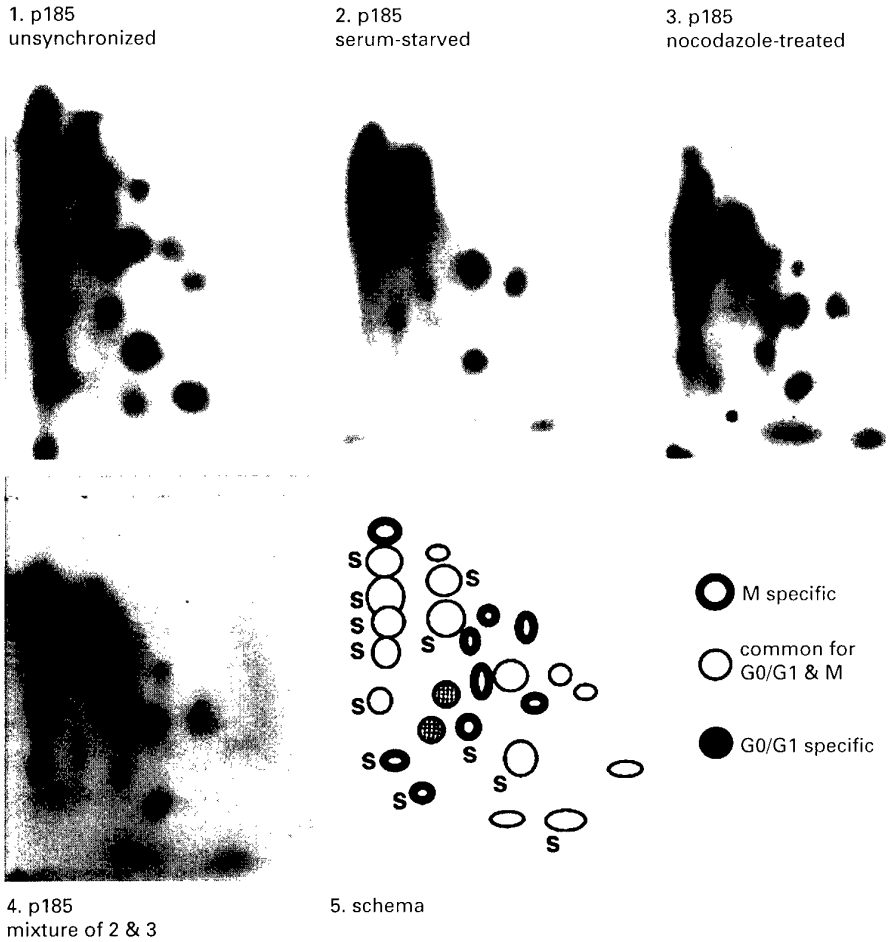

Figure 5 Phosphopeptide analysis of metabolically labeled p185^{neu} in G_0/G_1 or M phase. The p185^{neu} proteins from DHFR/G8, metabolically labeled with γ-^{32}P from unsynchronized (panel 1), serum-starved (panel 2), or nocodazole-treated (panel 3) cells were purified by immunoprecipitation, and the individual samples or an equal mixture from panel 2 and 3 (panel 4) were separated by SDS–PAGE gel and analysed for the presence of phosphopeptides as described in the Materials and methods. Individual phosphopeptide spots from the panel 3 were cut out, and phosphoamino acid analysis identified several phosphoserine residues (S), as shown in schema (panel 5). Two independent experiments were done to confirm these results

Feedback inhibition of p185^{neu} tyrosine kinase activity by PKC-mediated serine/threonine phosphorylation has also been reported (Cao *et al*., 1991; Dobashi *et al*., 1989). Although our results suggested the involvement of cdc2 in the phosphorylation of p185^{neu}, they do not rule out the involvement of other kinases in the process. The presence of additional phosphopeptides, several of them containing phosphoserine residues, in *in vivo* -labeled DHFR/G8 cells in M phase (Figure 6) already indicates this possibility. HER2/*neu* is phosphorylated *in vitro* on a single site in the cytoplasmic tail at threonine 1172 by the calmodulin-dependent protein kinase II (Feinmesser *et al*., 1996). The consensus sites of cdc2 (basic-Ser/Thr-Pro-polar-basic or basic/polar-Ser/Thr-Pro-X-basic) are, in fact, quite similar to those of MAP kinase (Pro-X-Ser/Thr-Pro), another Ser/Thr kinase, where X represents any amino acid present in proteins (Langan *et al*., 1989; Lin *et al*., 1991; Shenoy *et al*., 1989). In addition, the platelet-derived growth factor receptor (Yarden *et al*., 1986) and fibroblast growth factor receptor (Lee *et al*., 1989) also contain Ser-Pro motifs in the region C terminal to the kinase domain, although they lack the acidic region upstream from this sequence. Serine/threonine phosphorylation is likely to be a general phenomenon since a number of growth factor receptors could be phosphorylated by a proline-directed kinase in this region. It would be interesting to analyse the action of various serine/threonine kinases and their involvement in and relative contribution to negative regulation of receptor tyrosine kinases in general and the cell cycle in particular.

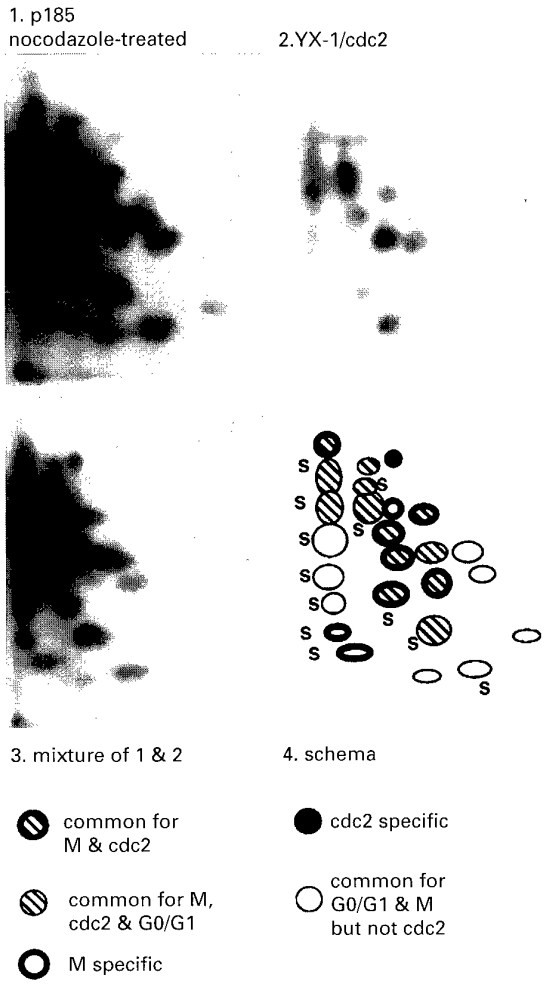

Figure 6 Comparison of the patterns of phosphopeptides and phosphoamino acids obtained from the *in vitro* and *in vivo* phosphorylation of p185^{neu} protein. DHFR/G8 cells were metabolically labeled after treatment with nocodazole and analysed as described in Figure 5 and the phosphopeptide spots are shown in panel 1. The recombinant-GS-p185^{neu} fusion protein (YX-1) was phosphorylated and analysed as described in Figure 4 and shown in panel 2. The phosphopeptide pattern obtained after mixing the samples from panels 1 and 2 are shown in panel 3. Phosphoamino acid analysis of individual spots from panel 3 revealed the prescence of several phosphoserine-containing peptides (S) as shown in the schema (panel 4). These results were confirmed in another independent experiment

Materials and methods

Cell culture and synchronization

Cell lines that express the normal p185^{neu} (DHFR/G8) and the mutation-activated p185^{neu*} have been described (Hung *et al*., 1986; Kiyokawa *et al*., 1995; Padhy *et al*., 1982). NIH3T3 cells were transfected with *ras*, *src* and *abl* oncogenes (NIH/*ras*, NIH/*src*, NIH/*abl*) as described earlier (Matin and Hung, 1994). Cells were grown in Dulbecco's modified Eagle medium : nutrient mixture F-12 (DMEM/F12 1 : 1) (GIBCO) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cells at half-confluence were starved in DMEM/F12 with 0.5% FCS for 48 h to arrest them in G_0/G_1 phase (Welch and Wang, 1993). To accumulate in pre-S phase and G2 phase, cells were incubated with aphidicolin and Hoechst 33342, respectively, as described (Kiyokawa *et al*., 1995). To synchronize the cells in M phase, they were incubated with DMEM/F12 containing 10% fetal calf serum and nocodazole (0.4 μg/ml) for 12–24 h, afterwhich cells that had a highly rounded mitotic shape were collected by mechanical shake-off. More than 90% of the nocodazole-treated shaken off cells were tetraploid according to DNA content analysis (Chackalaparampil and Shalloway, 1988). The adherent cells after nocodazole treatment were considered to be in G2/M phase.

DNA content analysis

For DNA content analysis, trypsinized cells were fixed for 30 min at −20°C in 70% ethanol-30% PBS mixture. After staining with PBS containing 5 μg/ml propidium iodide and 8 μg/ml RNAse A, cells were analysed on an EPICS PROFILE flow cytometer (Coulter) as described previously (Chackalaparampil and Shalloway, 1988). The results were analysed with the MultiCycle computer program (Pheonix Flow System).

Antibodies

Anti-cdc2 monoclonal antibody p34 (Santa Cruz Biotechnology Inc.,) was used for both immunoprecipitation and immunoblotting. Anti-p185^{neu} monoclonal antibody raised against the c-terminal (Ab-3) or the extracellular portion (Ab-4) and a rabbit antibody raised against the kinase domain (Ab-1) were obtained from Oncogene Science.

Generation of GST-p185^{neu} fusion proteins

cDNA fragments that correspond to different parts of the intracellular portion of rat p185^{neu} were amplified by PCR from pSV2neu and subcloned into pGEX-3X bacterial

expression vector (Pharmacia) at *Hind*III and *Sma*I sites. The GST-fusion proteins, YX-1 representing amino acids 934–1260 and YX-3 representing amino acids 681–993 of p185^{neu}, were produced in bacteria and purified.

Immunoprecipitation and Western blotting

After each treatment, cells were solubilized for 30 min on ice in lysis buffer (20 mM Na_2PO_4, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 mM NaF and 2 mM Na_3VO_4) and the total protein concentration was determined using the Bio-Rad protein assay kit. Electrophoretically separated proteins were transferred to a nitrocellulose membrane for 1 h at 3 mA/cm^2 using a semidry transblot system (Bio-Rad). After being blocked with PBS containing 5% milk, membranes were incubated with primary antibodies for 1 h at room temperature in 3% BSA/PBS, then washed with 0.05% Tween 20 in PBS. After 30 min of incubation with an appropriate secondary antibody conjugated with horseradish peroxidase and a subsequent washing with PBS-0.05% Tween 20, immunoblots were developed by the enhanced chemiluminescence reagent (ECL, Amersham) for 1 min and exposed to film (Hyper-film, Amersham). For immunoprecipitations, 500 μg to 1 mg of cell lysates were incubated with 1 μg of antibody and 50 μl of 50% protein-A agarose for 1 h at 4°C. After several washes with 0.05% Tween 20/PBS, immunoprecipitates were separated by electrophoresis and analysed.

Chemical crosslinking

Cells synchronized in the G_0/G_1 or M phase were washed with PBS twice and incubated with 1 mM bis(sulfosuccinimidyl) suberate (BS^3) in PBS for 30 min at 4°C, with rocking. The cells were solubilized with PI/RIPA buffer (Weiner *et al*., 1989).

In Vitro *immune complex kinase assay*

Commercially available cdc2 complex (Upstate Biotechnology Incorporated, New York) purified from the sea star *Pisaster ochraceus* was mixed with either the immunoprecipitated p185^{neu} proteins or the recombinant GST-p185^{neu} proteins. After being washed four times, once with PBS, twice with 50 mM Tris-HCl (pH 7.5) containing 0.5 M LiCl and once with kinase assay buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.5), the immunoprecipitates were incubated for 20 min at room temperature in 50 μl of kinase buffer, with 10 μCi of [^{32}P]γ-ATP (specific activity >3000 Ci/mM; Amersham). Reactions were stopped by adding 10 μl of 6×SDS-sample loading buffer. After separation on a 7% SDS–PAGE gel, phosphorylated proteins were visualized with autoradiography.

^{32}P metabolic labeling

Cells were metabolically labeled with ^{32}P-orthophosphate (500 μCi/ml, carrier-free; Amersham) for 8 h in phosphate-free DMEM supplemented with either 0.5% or 10% dialyzed FCS (Epstein *et al*., 1990).

Phosphopeptide and phosphoamino acid analysis

In vitro or *in vivo* ^{32}P-labeled p185^{neu} proteins were excised and extracted from SDS–PAGE gel as described previously (Cooper *et al*., 1983). One portion was digested with TPCK-treated trypsin and the peptides were separated on TLC plates by electrophoresis (horizontal direction, anode on left) in pH 3.5 buffer and chromatography (vertical direction) as described previously (Kiyokawa *et al*., 1995). Another portion was hydrolyzed in 6 N constantly boiling HCL for 60 min at 110°C and separated on TLC plates with unlabeled phosphoamino acid standards by two-dimensional electrophoresis for phosphoamino acid analysis. In both cases, phosphopeptides and phosphoamino acids were detected by autoradiography and the positions of phosphoamino acids were visualized by ninhydrin staining.

Acknowledgements

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Degradation of Estrogen Receptor by Emodin, a tyrosine kinase inhibitor, through Proteasome Pathway*

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Running Title: **Emodin induces estrogen receptor degradation**

Key words: **emodin, estrogen receptor, protease inhibitors, hsp90**

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[1] The abbreviations used are: ER, estrogen receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; hsp90, heat shock protein 90.

ABSTRACT

In an attempt to study the involvement of tyrosine kinase pathway in estrogen receptor signaling pathway, we used emodin, a tyrosine kinase inhibitor, to treat MCF-7 cells, a breast cancer cell line which express high level of estrogen receptor protein. Here we reported that emodin inhibited estrogen-induced expression of bcl-2 protein in MCF-7 breast cancer cells. Unexpectedly, treatment of the cells with emodin rapidly depletes cellular levels of estrogen receptor protein in a dose- and time-dependent manner. The pulse chase experiment showed that the decrease was resulted from enhanced degradation of estrogen receptor protein, not the rate of synthesis. To examine the mechanism involved in the emodin-enhanced degradation of estrogen receptor, inhibitors of the lysosomal (chloroquine), proteasome (PSI and MG115), and calpains pathways were used. We found that only PSI and MG115, which specifically inhibit the chymotrypsin-like activity of proteasome, blocked emodin induced depletion of estrogen receptor protein levels. The results suggest that the proteasome proteolytic pathway may be involved in the emodin-induced decreases in estrogen receptor protein levels. We then examined the effect of emodin on the hsp90-estrogen receptor heteromeric complex formation. We found that there was a marked increase in the complex formation. The data demonstrate that emodin may inhibit the dissociation of hsp90 from estrogen receptor, resulting in the degradation of estrogen receptor. Overall these findings indicate that proteasome-mediated protein degradation can modulate estrogen receptor protein level, hsp90 may involve the degradation of the estrogen receptor, and the possible use of emodin in the therapeutic manipulation of this process.

INTRODUCTION

The estrogen receptor belongs to a superfamily of nuclear receptors. It is a 66 kDa, ligand-activated transcription factor. After binding to its ligand, estradiol, the receptor forms homodimers and binds to DNA to regulate the gene expression, such as the bcl-2 protein, and progesterone receptor (1,2). Tyrosine kinase pathway has been implicated in the estrogen receptor pathway. In a study, treatment of tyrosine kinase inhibitor, tyrophostin, could suppress the estrogen-stimulated growth in MCF-7 cells, an estrogen receptor positive breast cancer cell line (3). Another study has shown that EGF via MAP kinase pathway caused the phosphorylation of a serine residue 118 which appears to be required for AF-1 domain activity (4). Furthermore, src, a tyrosine kinase, has been demostrated to be able to form complex with estrogen receptor in another estrogen receptor positive cell line, T47D (5). In addition, the estrogen receptor can be modified by phosphorylation on tyrosine residues, in particular Y537 in the human protein (6). Y 537 is located at the N-terminus of helix 12 in the hormone binding domain. The modificaiton at the tyrosine residue may represent an alternative mechanism for ligand independent activation of the estrogen receptor. All these data indicate that tyrosine kinase pathway interacts with the estrogen receptor pathway. However, the detailed mechanisms and which growth factor and or kinase pathway responsible for the phosphorylation and activation of the estrogen receptor at this position is still dubious.

The proteasome pathway is a major pathway of proteolysis in eukaryotic cells and have been shown to involve in regulating the turnover of several proteins, such as the tumor necrosis receptor (7), growth hormone receptor (8), immnoglobulin E receptor proteins (9), p53 (10), Mat-α2 repressor (11), and cyclins (10,12-14). Several molecules of ubiquitin are covalently linked to the target proteins in an ATP dependent manner. The ubiquitinated proteins are then recognized and subsequently destroyed by multisubunit proteasome comlex. In a recent report, it is shown that the rat uterine estrogen receptor

becomes ubiquitinated upon stimulation with its ligand (15). The findings strongly suggest the cellular protein level of estrogen receptor may be regulated by proteasome proteolytic pathway.

The estrogen receptor has been shown to form heteromeric complexes with different members of heat shock protein family, hsp90 and hsp70 as well as other proteins (16). The hsp90 is believed to maintain the ligand-free steroid receptor in a certain conformation so as to be responsive to the ligand stimulation. The hsp90-steroid receptor heterocomplexes are in a steady state of dynamic equilibrium of assembly /disassembly cycle (17). Upon binding to its ligand, estrogen receptor ligand binding domain has been suggested to have a conformational change that causes the release of hsp90 from the receptor, thereby exposing regions necessary for DNA binding, homodimerization, and nuclear localization. A recent report has shown that hsp90 may have another function as a quality control system in the refolding or degradation of some proteins (18). They showed that hsp90 can mediates the refolding of thermally denatured proteins *in vivo* and *in vitro*. However, with the addition of benzoquinone ansamycin, herbamycin A, originally classified as a tyrosine kinase inhibitor but shown to bind to hsp90, hsp90 was inhibited from normal dissociation with the protein and resulted in the degradation of the protein. Another group has demonstrated that hsp90 selectively recognizes destabilized mutant estrogen receptor proteins (19). These data suggest that hsp90 may be invovled in the maintenance of the receptor stability.

We previously showed that emodin can inhibit c-erbB-2 tyrosine kinase activity and preferentially suppresses growth and induces differentiation of the c-erbB-2 overexpressing breast cancer (20,21). Emodin is extracted from the roots of *Polygonum cuspidatum*, which has been used for the treatment of suppurative dermatitis, gonorrhea, favus, athletes foot, and hyperlipidemia in Oriental traditional medicine. It was also shown to inhibit the activity of $p56^{lck}$ kinase (22). In the present study, we examined the involvement of tyrosine kinase pathway in the estrogen receptor signaling by using emodin. However,

besides being as a tyrosine kinase inhibitor, we report here that by an unknown mechanism, emodin can cause rapidly degradation of estrogen receptor protein by proteasome proteolytic pathway.

EXPERIMENTAL PROCEDURES

Cell Culture-- MCF-7, T47D, and ZR-75-1 which are estrogen receptor positive cell lines were used. They were maintained in phenol red Dulbecco's modified Eagle's/F12 (GIBCO, Grand Island, NY) medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37° C under 5% CO_2 in air. For experiments requiring estrogen depleted condition, cells were incubated in phenol red free Dulbecco's modified Eagle's medium supplemented with 1% charcoal stripped serum for one day before initiation of the experiment. The charcoal stripped serum was prepared as described previously (23).

Reagents--- Estrogen (0.1μM stock solution) (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and stored at -20°C for up to one month. Emodin, genistein, *N*-Benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal (PSI), and carbobenzoxyl-leucinyl-leucinyl-norvalinal-II (MG115) were purchased from Sigma Co (St.Louis, MO) and RG13022 (Biomol, Plymouth Meeting, PA) were dissolved in DMSO. Chloroquine and EGTA (Sigma Co., St. Louis, MO) were dissolved in PBS. L-[^{35} S]Methionine was from Amersham (Arlington Heights, IL). Anti-bcl-2 antibody and anti-ubiqutin antibody were purchased from PharMingen and Sigma Co. Antiestrogen receptor antibody (SRA 1010) was obtained from StressGene (Vancouver, BC). Anti-estrogen receptor (D75) (24) and anti-hsp90 (AC88) (25) were generous gifts from Dr. G. Greene and Dr. David Toft respectively.

Western Analysis-- MCF-7 cells were treated with emodin (40 μM) for different time intervals. The cells were lysed with RIPA buffer (20 mM Na_2PO_4, pH 7.4; 150 mM NaCl; 1% TritonX-100; 1% aprotinin: 1mM phenylmethylsulfonyl fluoride; 10mg/ml leupeptin; 100mM NaF; and 2mM Na_3VO_4). The protein content was determined against a standard control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 80-100 μg of total protein lysates were used for SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to a nitrocellulose membrane. The membranes were blocked for 1h in 5% non-fat dry milk/ Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). The membranes were incubated with he primary antibodies, anti-estrogen receptor antibody (1:1000; D75) for detection of estrogen receptor protein or anit-actin antibody for detection of actin for equal loading at 4°C overnight. The membranes were then incubated with the goat-anti-rat antibody (1:2500 diluation) (Sigma Co., St. Louis, MO) or with the HRP-goat antimouse antibody (1:10,000 dilution) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for about 30 minutes at room temperature. The subsequent detection was done with enhanced chemiluminescence (ECL) system (Amershan Corp., Arlington Height, IL). For the dose responsiveness, MCF-7 cells were treated with different concentration of emodin for 4 hours. Then the estrogen receptor protein levels were examined by immunoblotting analysis as described above. For detection of the effect of protease inhibitors on emodin enhanced depletion of estrogen receptor protein, MCF-7 cells were treated with emodin (40 μM) and the protease inhibitors simultaneously for 2 and 4 hours. The controls were treated with the vehicles (DMSO and PBS) alone. The preparation of total protein lysates and subsequent immunoblotting analysis were performed as described above. The intensity of the protein was quantitated by NIH Image. The results were calculated as the percentage of the controls and normalized with actin.

Metabolic labeling of protein, immunoprecipitation and gel electrophoresis.-- MCF-7 cells were incubated for 1 h at 37°C in methionine-free medium containing 5 % dialyzed, heat-inactivated FCS, with or without emodin (40 μM). [^{35}S] methionine was added to yield 100 μCi/ml in the medium. Incubation was continued for an additional hour. Cells were then rinsed twice with warm, complete medium. Cells were incubated in the presence or absence of emdoin (40 μM) at various intervals after the chase, labeled cells were lysed in RIPA-B lysis buffer. Anti-estrogen receptor antibody (D75) immunoprecipitation was performed from 500 μg total cellular protein overnight at 4°C. 20 μg of rabbit anti-rat antibody was added to the mixture and incubated for 45 minutes at 4°C. Protein A-agarose was added and incubated for 45 minutes at 4°C. The mixture was washed three times with cold PBS. Immunoprecipitates were run on 6% SDS-polyacrylamide gels. The gel was fixed with 10% acetic acid/30% methanol for at least an hour and was then put into an enhancing solution for 1 hour. The gel was then dried at 80°C for at least 6 hours. The radiolabeled proteins were visualized by autoradiography. The intensity of the proteins was quantitated by NIH Image. Results were calculated as the percentage of the controls measured at the start of the chase period.

Co-immunoprecipitation-- Cellular protein was prepared by lysing the MCF-7 cells treated with 40 μM of emodin for various intervals in RIPA buffer with addition of 10 mM sodium molybdate. Immunoprecipitation with the monoclonal anti-estrogen receptor antibody SRA1010 (Sressgene, Vancouver, BC) was carried out. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with anti-hsp90 antibody (AC88) at 1:250 dilution. The membrane was stripped and reprobed with anti-estrogen receptor antibody (D75) for detection estrogen receptor.

RESULTS

Emodin Inhibits Estrogen-induced bcl-2 protein in MCF-7 cell--It is known that estrogen can induce the expression of bcl-2 protein in breast cancer cells (1,2). We therefore use the expression of bcl-2 protein as a marker to examine the effect of emodin on the estrogen receptor signaling pathway. We treated MCF-7 cells with different concentrations of emodin for 20-24 hours along with estrogen stimulation. As shown in Fig 1, the bcl-2 protein level was increased after estrogen stimulation. However, the bcl-2 protein induction was inhibited with the increasing doses of emodin. As previously reported, emodin is a tyrosine kinase inhibitor. It can inhibit $p56^{lck}$ as well as c-erbB-2 tyrosine kinase activity. Therefore, we examined the effect of emodin on the tyrosine kinase phosphorylaton of the MCF-7 cell proteins. The western blotting analysis of total protein tyrosine phosphorylation showed that there was general reduction of phosphorylation in cells treated with emodin (data not shown).

Depletion of Estrogen Receptor Protein by Emodin--The inhibitory effect of emodin on estrogen-induced bcl-2 protein can be explained by either emodin down-regulate the levels of estrogen receptor in MCF-7 cells or emodin inhibits tyrosine kinase activity involved in estrogen receptor signaling pathway. To test the first possibility, we treated the MCF-7 cells with 40 μM of emodin at different time intervals and then examined the protein levels by western blotting analysis. As shown in Fig. 2*A*, emodin could reduce the estrogen receptor protein level rapidly. The estrogen receptor protein level reduced to about 50% of the control after treating the cells with emodin for two hours (Fig. 2*B*). Then, we treated the MCF-7 cells with different concentrations of emodin for 4 hours. As showed in Fig 2*C* and *D*, the depletion of estrogen receptor protein levels follows a dose-dependent manner. To examine whether the emodin-induced depletion of estrogen receptor is a general phenomenon, we treated ZR 75-1 and T47D cells which are known to express

estrogen receptor protein with 40 μM emodin at different time intervals. Emodin could also enhance the depletion of estrogen receptor protein in both ZR 75-1 and T47D cell lines (data not shown). Taken together, the results indicate that emodin can rapidly deplete of estrogen receptor protein in different estrogen receptor positive cell lines.

We then examined whether different tyrosine kinase inhibitors could also deplete the estrogen receptor protein level. We treated MCF-7 cells with emodin (40 μM), genistein (100 μM), and RG13022 (5 μM) for 18 hours. As shown in Fig.3, both genistein and RG13022 could also significanly reduced the estrogen receptor protein level. These data suggest that tyrosine kinase pathway may be involved in the regulation of the estrogen receptor protein level.

Decreased stability of Estrogen Receptor protein in MCF-7 after Emodin treatment.-- The effect on estrogen receptor was not a consequence of cytotoxicity because MCF-7 cell morphology, as well as viability as assessed by trypan blue exclusion, were not affected after 4 hour treatment of emodin. The observed decreased estrogen receptor protein level by emodin could be explained either by diminished protein synthesis or by enhanced protein degradation. To distinguish these two possiblities, we performed a pulse-chase experiment. The cells were labeled with [^{35}S] methionine for 1 hour and chased for 1 to 4 hours in the presence or absence of emodin. As shown in Fig. 4, the turnover of the newly synthesized estrogen receptor was strikingly enhanced by emodin. The reduction of estrogen receptor level in the pulse chase experiment is consistent with the time course of the decrease of estrogen receptor protein levels as shown in Fig. 2. Taken together, these results demonstrate that the reduction in estrogen receptor protein levels by emodin is not because of diminished protein synthesis but of markedly increase in protein degradation.

Emodin-induced Estrogen receptor degradation involves the proteasome-- A previous report showed that the rat estrogen receptor was rapidly ubiquitinated after

estradiol stimulation (15). It suggests that 26S proteasome proteolytic pathway may involve in estrogen receptor degradation. To test whether the estrogen receptor protein can be regulated by the proteasome, MCF-7 cells were stimulated with estradiol in the presence or absence of the peptide aldehyde proteasome inhibitor, carbobenzoxyl-leucinyl-leucinyl norvalinal II (MG 115). The protein was extracted and quantified by western blot analysis. As shown in Fig.5 , there was a decrease of estrogen receptor level after estrogen stimulation. However, with the addition of the proteasome inhbitor, the estrogen receptor is protected from degradation. These results indicate that estrogen receptor protein can be regulated by the proteasome pathway.

To further examine the possible involvement of proteasome proteolytic pathway in emodin-enhanced estrogen receptor protein depletion, inhibitors of different proteolytic pathways were added simultaneously with emodin. Then, the estrogen receptor protein levels were detected by western blotting analysis. As shown in Fig. 6. the receptor protein remained steady in the control cells but it decreased rapidly in cells treated with 40 μM emodin. The protein levels also decreased in cells treated with either chloroquine, a lysosomal proteolytic inhibitor, or EGTA, a calpains inhibitor. However, in cells treated with PSI and MG115 resulted in the striking suppression of emodin-enhanced estrogen receptor depletion. Taken together, the findings indicate that 26S proteasome proteolytic pathway involves in the regulation of estrogen receptor and emodin can enhance the protein degradation through this pathway.

Increase in estrogen receptor-hsp90 heteromeric complex formation after emodin treatment--The estrogen receptor is known to form heteromeric complex with 2 molecules of hsp90 and other proteins (16,26,27). The hsp90 is believed to maintain the ligand-free steroid receptor in a certain conformation so as to be responsive to the ligand stimulation.. The receptor-hsp90 heteromeric complexes are in a steady state of dynamic equilibrium of assembly /disassembly cycle (17). A recent report has shown that hsp90 may have a function as a quality control system in the refolding or degradation of some proteins (18).

We therefore examined whether emodin may affect the heteromeric complex formation between hsp90 and estrogen receptor in association with the receptor degradation. As showed in Fig.6, there was only small amount of hsp90-estrogen receptor heteromeric complex before emodin treatment. After incubating with 40 μM emodin, the amount of hsp90-bound estrogen receptor markedly increased (Fig. 7*A*). The level of cellular levels of hsp90 remained unchanged (Fig. 7*B*). The results imply that there is apparently only small amount of estrogen receptor bound to hsp90 under steady state conditions. However, emodin, by an unknown mechanism, inhibits the dissociation of the hsp90-estrogen receptor complex which may lead to the proteasomal degradation of estrogen receptor (Fig. 8).

DISCUSSION

Many different proteins have been shown to undergo proteasome proteolytic degradation, including p53 (10), and HER-2/neu (28). To our knowledge, it is the first report to demonstrate that the estrogen receptor can also undergo proteasome degradation. It is known that estrogen receptor transcription rate is decreased after estradiol stimulation. We here showed that following estradiol stimulation, the protein level of the receptor can also be downregulated via the proteasome. Furthermore, the estrogen receptor degradation can be induced by emodin. Emodin has been reported as tyrosine kinase inhibitor that represses the activity of $p56^{lck}$ kinase by competitively inhibiting the binding of ATP (22). We have also showed that emodin can repress the c-erbB-2 tyrosine kinase activity in c-erbB-2 overexpressing breast and lung cancer cell (20,21). In this study, we showed that emodin can cause rapid degradation of estrogen receptor protein through the proteasome degradation pathway. The mechanism of emodin-enhanced degradation of estrogen receptor remains to be investigated. Emodin, as a tyrosine kinase inhibitor, may inhibit some cellular tyrosine kinase(s) which may be important to maintain the folding of the

estrogen receptor, since other known tyrosine kinase inhibitors, namely genistein and RG13022 can also significantly reduce the protein level of estrogen receptor. In several reports, tyrosine residue at 537 of estrogen receptor can be phosphorylated and is important for the receptor dimerization and DNA (6,29-31). This tyrosine residue is located in the hsp90 binding region. It may be possible that tyrosine phosphorylatioin of the estrogen receptor may also be important for binding to hsp90. Emodin may decrease the tyrosine phosphorylation of the ER by inhibiting an unknown cellular tyrosine kinase, leading to changes in hsp90 binding and subsequent degradation. Another group of tyrosine kinase inhibitors, anasmycins, has showed to have the ability to deplete several different cellular proteins. Anasmycins can bind to hsp90 and enhancing the degradation of several different proteins, including raf-1, glucocorticoid hormone receptor, and c-erbB-2/p185 protein (18,28,32-34). However, we have previously showed that emodin does not decrease the level of c-erbB-2/p185 (20,21) under our experimental conditions. This suggests that emodin enhanced degradation may be through a mechanism different from that induced by anasmycins. Another possibility is that these two kinds of drugs may have the same mechanisms but different specificities.

The role of hsp90 in estrogen receptor is not well defined. The hsp90 may involve in maintaining the ligand-free receptor in such confirmation that it can respond to the ligand stimulation. Upon ligand stimulation, hsp90 will be released from the steroid receptor, thereby allowing the receptor to form homodimers, localize to the nucleus, and bind to DNA. Others have suggested that hsp90 may be necessary for the receptor stability. A recent report showed that hsp90 selectively binds to a pointed mutated estrogen receptor which has a destabilized ligand-binding domain, but not the wild-type estrogen receptor (19). The results suggest that hsp90 may be required for reduction of the levels of partially folded or misfolded estrogen receptor. Furthermore, another recent report has showed that hsp90 can be a quality control system which mediates protein folding or degradation (18). Upon binding to anasmycin, they showed that hsp90 shifts from refolding to degradation

mode by preventing its release from the substrate. Consistent with this notion, we here showed that only a small amount of estrogen receptor binds to hsp90 under the steady state conditions. After emodin treatment, there is an significant increased association of hsp90 and estrogen receptor while the receptor proceeds to degradation. Our results strongly support the idea that hsp90 can act as a quality control system capable of mediating degradation. Emodin, by an unknown mechanism, may inhibit the normal dissociation of the hsp90-estrogen receptor heterocomplex. The receptor will then signal to undergo the proteasome proteolytic degradation.

In summary, our findings indicate that proteasome-mediated protein degradation can modulate estrogen receptor protein level, and emodin can induce the receptor degradation probably via disrupting the dynamic equilibrium of the receptor-hsp90 complex. Emodin represents another class of drug acting on the estrogen receptor and could be potentially used in the therapeutic manipulation of this degradative process.

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FIGURE LEGENDS

Fig. 1. **Emodin inhibits estrogen-induced bcl-2 protein expression in MCF-7 cells.** MCF-7 cells were grown in the estrogen depleted medium for one day before the initiation of the experiment. The cells were then stimulated with estrogen (10 nM). Different concentrations of emodin were added simultaneously. After 24 hours, total cell lysates were prepared and immunoblotting analyses for bcl-2 protein were determined. The membrane was stripped and reprobed with anti-actin antibody to show the protein loading.

Fig. 2. **Depletion of estrogen receptor protein in estrogen receptor positive cells by emodin.** *A*, MCF-7 cells were exposed to 40 μM emodin for various time intervals and extracted as described in "Experimental Procedures". Estrogen receptor protein levels in MCF-7 cells were measured by immunoblotting with monoclonal antibody D75. The same membrane was stripped and reprobed with anti-β-actin antibody to show the protein loading. *B*, the proteins were quantitated by NIH Image software and plotted as the percentage control (without emodin) and normalized with actin. C, MCF-7 cells were incubated with different concentrations of emodin for 4 hours. The protein levels were then examined by immunoblotting as described above. The protein levels were quantitated as described above and plotted as showed in panel D.

Fig. 3. **Other tyrosine kinase inhibitors can reduce estrogen receptor protein level.** MCF-7 cells were treated with emodin, RG13022, genistein, or the solvent (DMSO) for 18 hours. Immunoblot analysis were carried out to detect the estrogen receptor protein level. The same membrane was reprobed with anti-actin antibody for loading control.

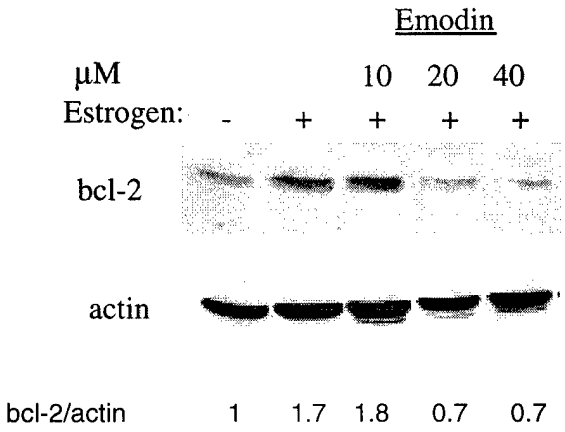
Fig. 4. **Emodin-enhanced estrogen receptor protein degradation.** Pulse chase experiment was performed to determine the stability of the estrogen receptor proteins after treatment with emodin (see "Experimental Procedures" for details). *A*, MCF-7 cells were treated with emodin at different time intervals. *B*, MCF-7 cells were treated with DMSO at various time intervals. *C*, proteins were quantitated by NIH Image software and plotted as the percentage of the value at the beginning of the chase.

Fig. 5. **Involvement of Proteasome Pathway in Estrogen-induced Estrogen Receptor degradation.** MCF-7 cells were stimulated with 10 nM estradiol in the presence or absence of MG 115 for 24 hours. For the control, ethanol and DMSO were added. The proteins were extracted and western blot analyses were performed to detect estrogen receptor and actin (for protein loading).

Fig. 6. **Effect of different protease inhibitors on emodin-induced estrogen receptor protein degradation.** Chloroquine (100 μM), EGTA (5 mM), MG115 (25 μM), and PSI (25 mM)were added to MCF-7 cells simultaneously with 40 mM emodin at several time intervals. PBS and DMSO were added to the control. The cells were then harvested and the expression levels of estrogen receptor protein were measured by western blotting analysis as described in Materials and Methods.

Fig. 7. **Enhanced association of hsp90 and estrogen receptor protein in MCF-7 cells after incubation with emodin.** Estrogen receptor immunoprecipitates (by anti-estrogen receptor antibody, SRA1010) from MCF-7 cells were analyzed by SDS/PAGE and immunobloting with anti-hsp90 (AC88). Normal mouse serum (NMS) was used instead of anti-estrogen receptor antibody as a control. The membrane was then stripped and reprobed with anti-estrogen receptor (D75) antibodies (*A*). The same protein lysates were used to examine the hsp90 protein level by immunoblotting (*B*).

Fig. 8. **A hypothelical model of how emodin induces estrogen receptor degradation.**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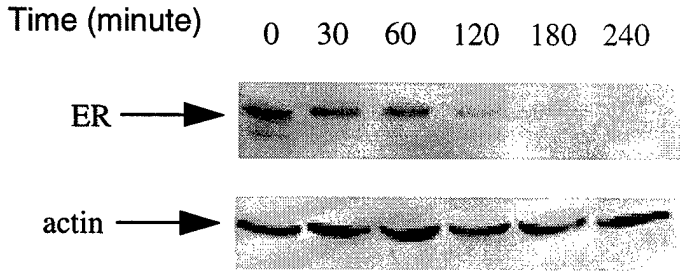
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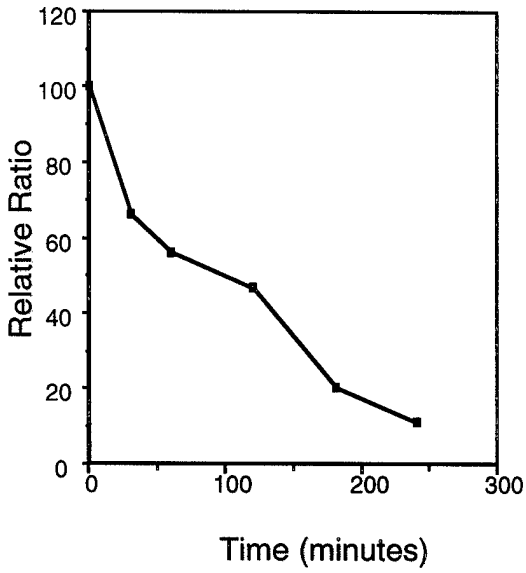
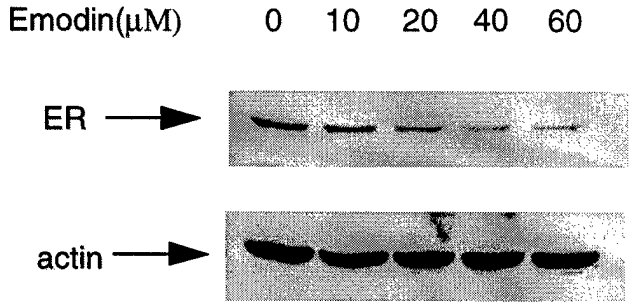
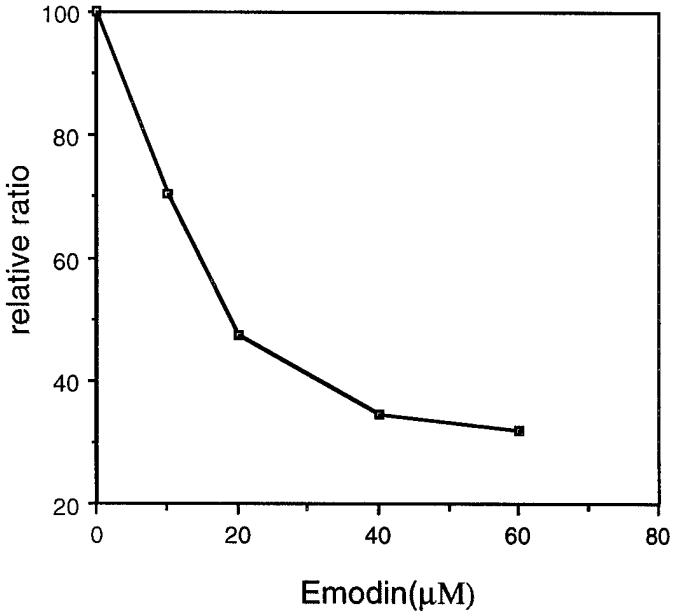
B

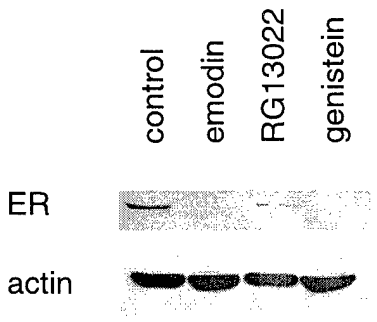

Relative Ratio
120
100
80
60
40
20
0
0
100
200
300
Time (minutes)

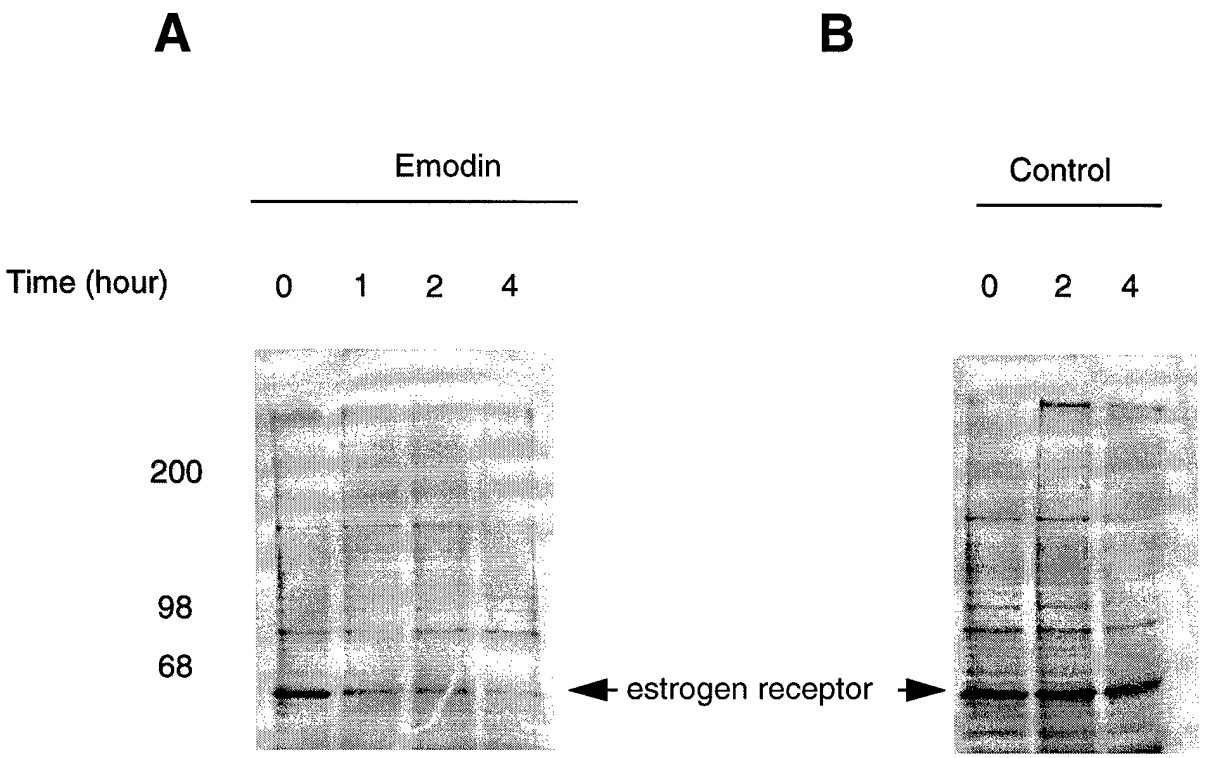
C.

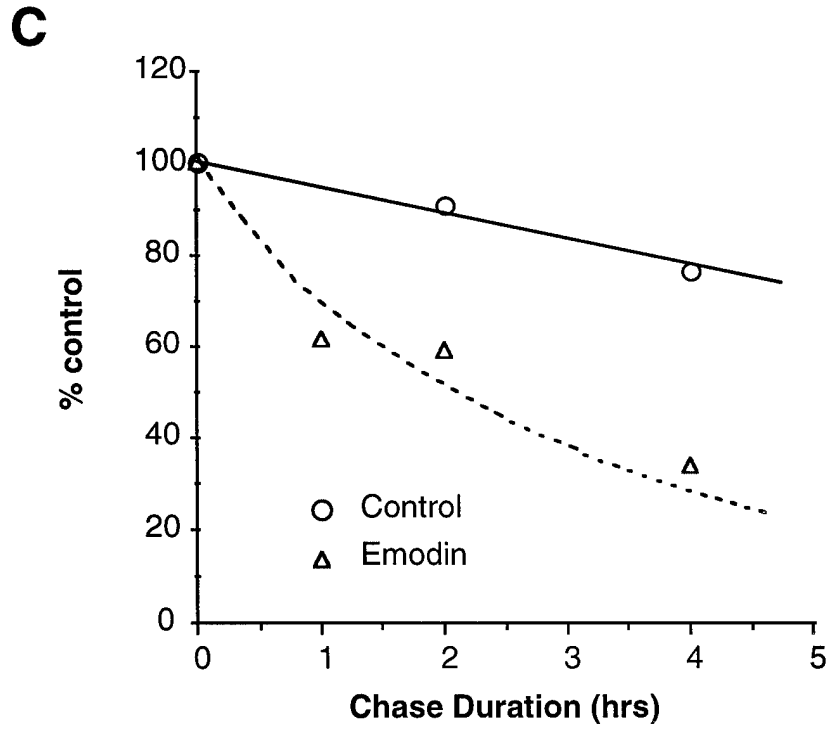

Emodin(μM)
0 10 20 40 60
ER
actin

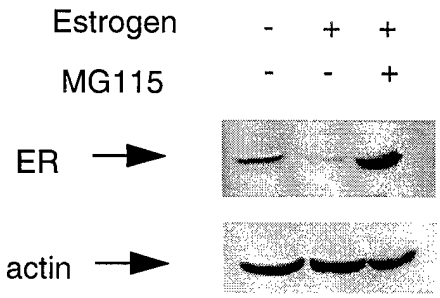
D.

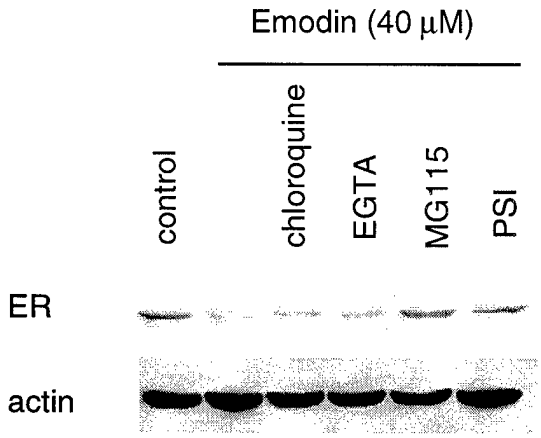

relative ratio
100
80
60
40
20
0
20
40
60
80
Emodin(μM)

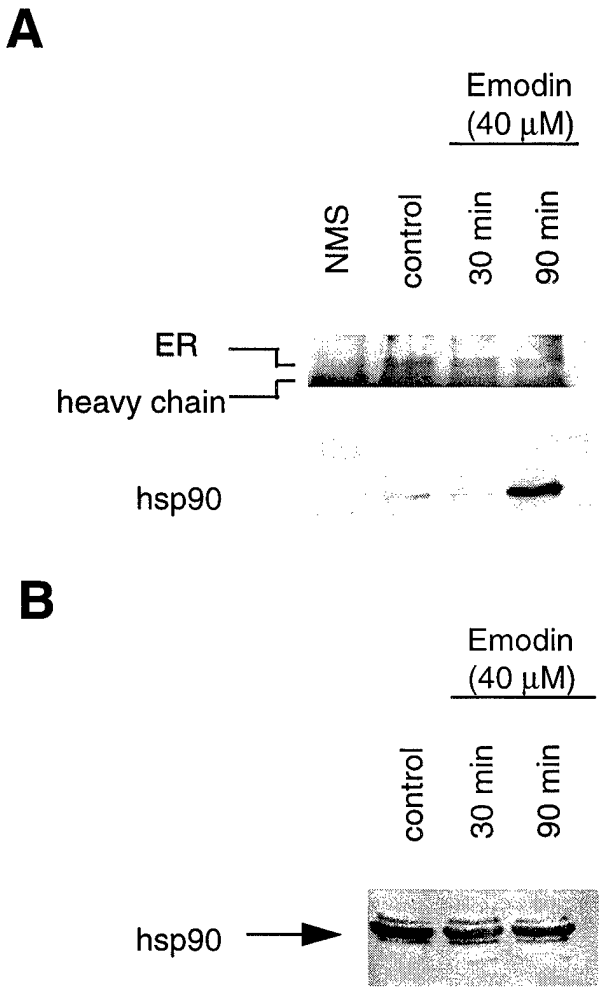

control
emodin
RG13022
genistein
ER
actin

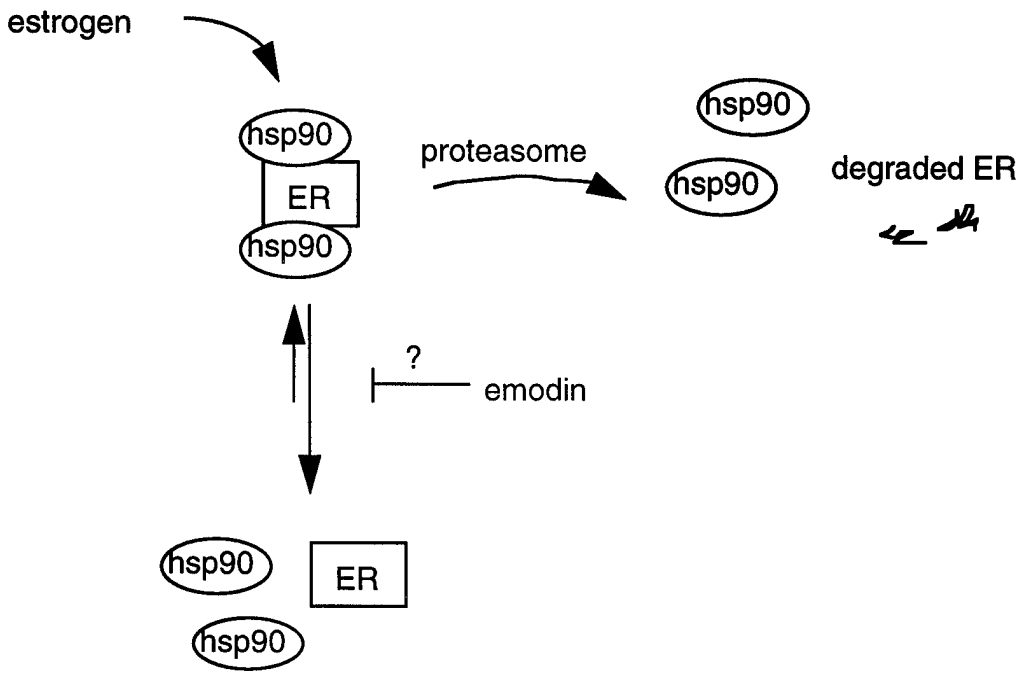

A
B
Emodin
Control
Time (hour)
0 1 2 4
0 2 4
200
98
68
estrogen receptor


C
120
100
80
60
40
20
0
% control
Control
Emodin
0 1 2 3 4 5
Chase Duration (hrs)


Estrogen
- + +
MG115
- - +
ER
actin


Emodin (40 μM)
control
chloroquine
EGTA
MG115
PSI
ER
actin


A
Emodin
(40 μM)
NMS
control
30 min
90 min
ER
heavy chain
hsp90
B
Emodin
(40 μM)
control
30 min
90 min
hsp90


estrogen
hsp90
ER
hsp90
proteasome
hsp90
hsp90
degraded ER
?
emodin
hsp90
ER
hsp90